

**Methods for genome-scale gene
perturbation studies of the TRAIL-
induced apoptosis pathway in mammalian
cell culture**

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Declaration

This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and acknowledgements. No part of this work has been submitted for any other degree at this or any University.

This dissertation does not exceed the page limit specified by the Biology Degree Committee.

Ian Sudbery, November 2007.

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Abstract

Modern techniques, particularly RNA interference, but also the systematic over-expression of full length open-reading frames (ORFs), have promised to allow traditional genetic screening paradigms to be transferred to mammalian cell culture systems in order to study medically relevant pathways and annotate function onto the genome.

TNF Related Apoptosis Inducing Ligand (TRAIL) induces apoptosis in many tumour cells, but not in the majority of normal cells. As such it has generated much excitement as a potential anti-cancer treatment. However, the molecular basis of the regulation of sensitivity to TRAIL is not fully understood. Here an assay for the sensitivity of HeLa cells to TRAIL is used to compare different approaches to RNAi screening. Various tests indicated that RNAi screening for novel TRAIL genes is feasible using siRNAs but not shRNAs.

RNAi screens were carried out using both a library of siRNAs targeting 901 Kinase and Phosphatases and a larger library targeting the “Druggable Genome”. Genes having the largest effect on TRAIL sensitivity were rigorously confirmed and controlled for off-target effects using multiple siRNAs and multiple assays. Thus eight novel genes involved in TRAIL-induced apoptosis were identified (Sharpin, MAST4, IKBKE, MAX, IGF1R, PDE11A, INADL and TEGT).

A thorough examination of the seed sequences of high scoring siRNAs revealed that several seed sequences were over-represented in high scoring siRNAs. This suggests that screening may enrich for siRNAs with relevant off-target effects. In addition comparison of these seed sequences to those of natural miRNAs identify four candidate miRNAs which may be involved in regulation of TRAIL-induced apoptosis.

A screen was also carried out to assess the effect of the over-expression of 288 full-length ORFs from chromosome 22. Several clones that have a reproducible effect on the sensitivity of cells to TRAIL were identified, although failure of these genes to have an effect in secondary assays mean that their physiological involvement in the pathway is unknown.

In conclusion, genome-scale systematic gene perturbation studies are powerful tools for annotation of gene function, and for isolating novel genes in medically relevant pathways, but they must be used with care and an awareness of their possible pitfalls.

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Abbreviations

5-FU	5-Fluorouracil
6mer	Hexamer
7mer-A1	Heptamer matching bases 2-7 of a mature miRNA with an additional A at position 1
7mer-m8	Heptamer matching bases 2-8 of a mature miRNA
8mer	Octamer
ADP	Adenosine DiPhosphate
ATP	Adenosine TriPhosphate
bp	Base Pair(s)
BSA	Bovine Serum Albumin
Casp8	Caspase-8
cDNA	Complementary DNA
cFLIP	Cellular FLICE Inhibitory Protein (also known as CFLAR)
CMV	CytoMegaloVirus
CNS	Central Nervous System
CNS-DCs	Central Nervous System DCs
COSMIC	Catalogue Of Somatic Mutations In Cancer
DAPI	4',6-DiAmidino-2-PhenylIndole
DcR1	Decoy Receptor 1 (also known as TNFRSF10C or TRAIL-R3)
DcR2	Decoy Receptor 2 (also known as TNFRSF10D or TRAIL-R4)
DCs	Dendritic Cells
DISC	Death Inducing Signalling Complex
DNA	Deoxyribose Nucleic Acid
DR4	Death Receptor 4 (also known as TNFRSF10A or TRAIL-R1)
DR5	Death Receptor 5 (also known as TNFRSF10B or TRAIL-R2)
dsRBP	Double Strand RNA Binding Protein
dsRNA	Double-Stranded RNA
EDTA	EthyleneDiamineTetra-acetic Acid
EMCV	EncephaloMyoCarditis Virus
esiRNA	endoribonuclease-prepared siRNA
EST	Expressed Sequence Tag

FACS	Fluorescent Activated Cell Sorting
FADD	FAS Associated Death Domain protein
FBS	Fetal Bovine Serum
FDR	False Discovery Rate
FTTC	Fluorescein IsoThioCyanate
Flp/FRT	FLiPase/Flipase Recombination Target
FWER	FamilyWise Error Rate
GDP	Guanosine DiPhosphate
GFP	Green Fluorescent Protein
GNF	Genomics Institute of the Novartis Research Foundation
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
GTP	Guanosine TriPosphate
H ₂ O	Water
H ₂ O ₂	Hydrogen Peroxide
IL-2	Interleukin-2
kb	KiloBase
LB	Luria-Bertani Broth
Log	Logarithm
LTR	Long Terminal Repeat
MAD	Median Absolute Deviation
MAPK	Mitogen Activated Protein Kinase
miRNA	MIcro RNA
mRNA	Messenger RNA
NF- κ B	Nuclear Factor-kappa B
NK (cells)	Natural Killer (cells)
NoT	Not Transfected
NPI	Normalised Percentage Inhibition
nt	NucleoTide
Oligo	Oligonucleotide
ORF	Open Reading Frame
ORFeome	The totality of all ORFs in an organism
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PGK	PhosphoGlycerate Kinase
PKC	Protein Kinase C
pSM2	pSHAG-MAGIC2
PTGS	Post Transcriptional Gene Silencing
qPCR	Quantitative PCR
qRT-PCR	Quantitative Reverse Transcription PCR
RdRP	RNA Dependent RNA Polymerase
Rep 1	Replicate 1
Rep 2	Replicate 2
RISC	RNA-Induced Silencing Complex
RLC	RISC Loading Complex
RLU	Relative Luminescent Units
RNA	Ribose Nucleic Acid
RNAi	RNA Interference
rpm	Revolutions Per Minute
RT-PCR	Reverse Transcription PCR
SCF	Skp, Cullin, F-box
shRNA	Short Hairpin RNA
shRNA ^{mir}	Short hairpin RNA with micro RNA based design
siRNA	Small Interfering RNA
ssRNA	Single-Stranded RNA
TNF	Tumor Necrosis Factor
TRAIL	TNF-Related Apoptosis Inducing Factor
UTR	UnTranslated Region
UV	UltraViolet (radiation)
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate

1 INTRODUCTION

The systematic creation of mutations, study of resulting phenotypes and identification of the genes responsible, or genetic screening, has proved a very powerful way of studying gene function in model organisms. Yeast geneticists are known to wax lyrical about “the awesome power of yeast genetics”. Genetic screens have, amongst other achievements, delineated the mechanism of cell-cycle control in yeast (Hartwell, Culotti & Reid 1970, Nurse, Thuriaux & Nasmyth 1976), identified the genes involved in embryonic development in flies (Nusslein-Volhard, Wieschaus 1980), and shown which genes are involved in programmed cell death in the worm (Ellis, Horvitz 1986). These studies, which go right to

the very heart of how cells function and how organisms are put together and work, earned those involved Nobel Prizes.

With the completion of the genome sequence of several organisms, including the human genome, and the prediction of the genes contained therein, one of the most important tasks for biologists today is understanding the function of each of the approximately 25,000 genes in the human genome and their roles in the disruption of normal cell operation in disease. Both forward genetic screens, where mutations causing a particular phenotype are associated with genes, and reverse genetic screens, where the phenotype of a defined mutation in a defined gene is studied, are powerful tools for adding functional annotation to the genome sequence. However, screening has thus far proved difficult in mammalian cell culture, due to the difficult and time-consuming nature of generating homozygous loss function mutations in a diploid, non-sexual system, and screens have mostly been restricted to gain-of-function, over-expression screens. Such over-expression screens can identify new gene function by expressing a gene in a situation where it would not normally be expressed, or by increasing its level leading to a change in the state of cellular pathways/networks and thereby revealing its role. This sort of screening is made more systematic by the availability of collections of clones representing full-length, sequence-verified open reading frames (ORFs), with exactly one clone representing each transcript in a genome, or a defined subset of a genome.

RNA interference (RNAi) is a relatively new method for “knocking-down” or reducing gene function, as opposed to complete loss of gene function produced by a gene “knock out” or deletion. First demonstrated by researchers in the worm *Caenorhabditis elegans* (Fire et al. 1998) and related to post-transcriptional gene silencing (PTGS) or co-suppression in plants (Napoli, Lemieux & Jorgensen 1990), this technique relies on the fact that the introduction of double-stranded RNA will trigger the degradation of complementary mRNA (Schwarz et al. 2002). Although it is true that RNAi results in a knock-down rather than a knock-out (a fact that can be an advantage in some cases) and that there are questions over the specificity of RNAi, possibly due to its overlap with the micro RNA pathway (see section 1.2.3), RNAi offers for the first time the possibility of genome-scale loss-of-function screening in mammalian cell systems.

RNAi screening in mammalian cell systems can be conducted in a number of ways, using a number of RNAi-inducing reagents, each of which has its strengths and weaknesses and each of which is suited to different tasks. Inducing RNAi with small interfering RNAs (siRNAs) is quick, efficient and reliable, but also transient and expensive. On the other hand,

bacterial clones carrying plasmids encoded short-hairpin RNAs (shRNAs) provide a limitless supply of reagent, and shRNAs can be used to generate stable or even conditional knock-down. The ability to recover and identify shRNAs at a later point also allows them to be used in pooled selections.

TNF-related apoptosis-inducing ligand (TRAIL) is a ligand that induces apoptosis in a subset of cancer cells, but not in normal cells (Walczak et al. 1999). However, the mechanism by which tumour cells are sensitive and normal cells are resistant is not fully understood. Understanding this mechanism allows us to increase the usefulness of TRAIL as an anti-cancer agent by being able to predict the sensitivity of cells, and also devise ways of sensitising insensitive tumour cells without sensitising normal cells. Furthermore, the understanding of a common weakness of diverse tumour cells over normal cells helps to understand the molecular basis of cancer itself, and may open the way for novel treatments based on this molecular Achilles' heel.

The TRAIL-induced apoptosis pathway presents a good candidate for genome-scale RNAi screening. Apoptosis is a simple and readily measurable phenotype which lends itself to screening by a number of different strategies. A number of genes involved are already known, which provides positive controls against which the performance of screens can be measured. Indeed, a small-scale screen for genes involved in the TRAIL-induced apoptosis pathway has already been successful in identifying new genes and new pathways involved (Aza-Blanc et al. 2003).

In this work, I will compare different methods for genome-scale screening of the TRAIL pathway, focusing on RNAi. Several large-scale screens for new genes in the TRAIL pathway will be described, along with confirmation of hits from these screens. Finally, effectiveness of the different screens and the usefulness of the results will be assessed.

1.1 Genetic Screening

Genetic screening is the practice of studying a particular biological process through the effects on that process of large numbers of genetic changes (mutations), and thereby identifying the genes involved. The term "mutation" can refer to the change in the organism or gene or the actual molecular change itself. At the organism level, mutations can be classified as hypomorphic (reduced gene function, of which a null mutation is most extreme example), hypermorphic (increased gene function) or neomorphic (generation of new functions for a gene). Of these, the most common generated by random mutagenesis are hypomorphic mutations. When considering screening two distinctions can be made: forward

and reverse genetic screens, and selective and non-selective screens.

Forward or traditional genetic screens start with a phenotype of interest and then generate more or less random mutations that change that process. The gene connected to mutations of interest must then be identified. The availability of complete genome sequences and the identification of all or at least many genes in these genomes allows the use of reverse genetics. A reverse genetic screen starts from the gene and then proceeds to assess the impact of mutating that gene.

The second distinction that can be drawn is between selective and non-selective screens. In a non-selective screen each mutant is assessed individually for its effects on the phenotype of interest. This can be very labour-intensive, particularly when mutants which give the phenotype of interest are expected to be rare. In a selective screen, a large number of mutants are exposed to a selective pressure such that mutants with the phenotype of interest are separated from the majority of uninteresting mutants or from wild-type cells or organisms.

1.1.1.1 Forward genetic screens in model organisms

Genetic screens and selections have been particularly successful when applied to model organisms. The vast array of screening paradigms and methodologies available in model organisms is outlined in the Nature Reviews Genetics series, “The Art and Design of Genetic Screens” (Casselton, Zolan 2002, Forsburg 2001, Grimm 2004, Jorgensen, Mango 2002, Kile, Hilton 2005, Patton, Zon 2001, Shuman, Silhavy 2003, St Johnston 2002).

The use of random mutagenesis by radiation or chemical mutagens in yeast and bacteria is greatly aided by the ability to grow these organisms as haploid clonal populations, as most often these methods lead to hypomorphic or null alleles which are likely to be recessive in heterozygous diploid organisms. Early screens in *Escherichia coli* delineated the repressor model of gene regulation in the *lac* operon (JACOB, MONOD 1961), and screens in bacteria continue to be useful for understanding the basic mechanisms of genetics; for example, screens for mutator genes defined the principles of DNA repair in bacteria.

The yeast is often seen as the screener’s organism of choice for studying processes in higher organisms that are conserved in this organism. This is attributable to the ease of genetic manipulation, the fact that it can be grown as diploids or haploids, its ability to reproduce sexually or clonally, its very short generation time, and particularly its high recombination rate. Nevertheless, it is a eukaryote whose fundamental cellular mechanisms are remarkably similar to those of higher organisms, so that the knowledge gained has a

general applicability. The ability to generate and manipulate large numbers of mutants allows the isolation of rare mutations such as temperature-sensitive mutants, which are phenotypically normal at one temperature, but show a mutant phenotype at another. This allows the maintenance of homozygous mutants of genes that would otherwise be very deleterious, a strategy that was employed in the seminal screens for genes involved in the control of the cell-division cycle (Hartwell, Culotti & Reid 1970, Nurse, Thuriaux & Nasmyth 1976).

In a forward genetic screen, once a mutation has been isolated, the gene responsible must be identified. The high recombination rate and availability of a sexual cycle mean that mutant genes can be identified by recombination mapping. Alternatively, loss-of-function mutants can be identified by transforming cDNA libraries into cells and screening for rescue of the phenotype, a process known as cloning by complementation.

Although yeast is easy to manipulate, there are many processes that cannot be studied in yeast, particularly those related to the development of an organism and the interaction of cells. Here, worms, fish and flies are the most common and powerful models. Mutations in these models can be generated by radiation, chemical mutagenesis or insertional mutagenesis using mobile elements. Generally, these models require multi-generational breeding schemes to generate the homozygous mutants need for screening for the effects of recessive mutations. Such F₃ recessive screens have included the identification of recessive mutations involved in the embryonic development of *Drosophila melanogaster* (Nusslein-Volhard, Wieschaus 1980). Worms have an advantage here as they reproduce hermaphroditically, which can simplify breeding schemes, reducing number of generations necessary to produce homozygous mutants. This sort of screening was used to identify genes involved in apoptosis in *C. elegans* (Ellis, Horvitz 1986). *C. elegans* is particularly well suited to the study of the genetic basis of development because of its invariant cell lineage (Sulston, Horvitz 1977). The Zebrafish (*Danio rerio*) can be induced to grow as haploids for the first three days of life, or can be induced to reproduce parthenogenetically (Streisinger et al. 1981).

As in yeast and bacteria, once mutants have been isolated, the genes responsible must still be identified, a much slower process than in yeast owing to the longer generation times involved. Insertional mutagenesis can help here as the insertion of an element into the genome tags the insertion site, sequences of which can be recovered by PCR and sequencing, and then mapped back on to the genome of a sequenced species.

Screening in the mouse is technically possible, but is slow and expensive since large numbers of homozygous mutant mice must be generated, and the genes responsible must be

mapped through recombination mapping, again requiring the crossing and examination of many mice. There are several methodologies that can speed up this process, such as the use of balancer chromosomes (Kile et al. 2003) or deletion strains (Rinchik, Carpenter & Selby 1990) for screen regions of the genome. An example of a genome-wide screening effort is the identification of genes involved in the innate immune system (Hoebe et al. 2003).

1.1.1.2 Screens vs. Selections

Screening as described above requires the examination of many individuals to identify mutants which match the phenotype in question. Selections can speed up the process by eliminating the need to examine every individual; they are particularly useful in isolating very rare events. Selections are common in yeast and bacteria where large numbers of mutants can be generated and maintained together and then a selective pressure applied such that only mutants of interest will be recovered. Selections are not however, only applicable to yeast and bacteria. Sydney Brenner used resistance to the drug alicarb to select for genes involved in the synapse function (Brenner 1974) in *C. elegans*.

The processes that can be studied by selections are different from those that can be studied using screens. Almost any phenotype can be studied using a screen, but selection restricts this. Firstly, only phenotypes that allow separation can be used. Secondly, in screens phenotypes can be measured in a quantitative way. However, in a selection genes are either isolated or not, and devising paradigms that allow for quantitative assessment of mutants is much more difficult. This is particularly important as the current paradigm for understanding cellular function shifts away from that of a pathway, where the presence of each component is required for the functioning of the pathway, to a network paradigm where each component has quantitative effect on the phenotypic output of the network.

1.1.1.3 Modifier screens

Modifier screens attempt to find mutations in second genes which alter the phenotype of initial mutants. Modifier screens can be divided into two categories: screens for mutations which enhance the phenotype of the initial mutation (Enhancer screens), and screens for mutations which suppress the phenotype of the initial mutation (suppressor screens). Such screens are useful for identifying genes which are involved in the same pathways as the gene carrying the original mutation. Enhancer screens can also make recessive hypomorphic alleles dominant, as under normal circumstances a 50% reduction in gene activity seen with a heterozygous hypomorphic allele may be able to support a wild-type

phenotype, but cannot when paired with another mutation in the same pathway. This is particularly useful if a strong mutant in a gene is lethal. In this way the genes downstream of the Sevenless (*sev*) gene in flies were identified. *Sev* is a receptor tyrosine kinase involved in eye development. A weak hypomorph of *sev* can support eye development, but only just. Screening for genes which disrupted eye development in a weak *sev* background isolated Son of Sevenless (SOS), which is a Ras guanine exchange factor (RasGEF) that activates Ras by increasing the rate of exchange of the GDP bound to inactive Ras for GTP (Simon et al. 1991).

Suppressor screens work in the opposite way. Lackner *et al* screened worms that had an activating mutation in the *C. elegans* RAS homolog *let-60* for mutations which suppressed the multi-vulval phenotype of this mutant and gave phenotypically normal worms (Lackner et al. 1994), identifying the MAP kinase homologue *mpk-1* as being downstream of RAS.

1.1.1.4 Saturation and the limitations of screening

The Nusse-Volhard screen for genes essential for embryo development is an important example as it was an attempt at “saturation screening”. In a saturating screen, the aim is to generate enough mutants that every gene in the genome has been mutated and so, in theory, identify all genes involved in a process. A screen is generally said to be reaching saturation when the same genes are identified multiple times. However, such screens are unlikely to ever identify all genes involved in a process for a number of reasons. Firstly, the chance of mutating a target is related to its size. Small targets are unlikely to be hit. For example, micro RNAs (miRNAs) such as *lin-4* in the worm have active sequences of only 22nt, and although *lin-4* is important for developmental timing, only two alleles have ever been identified. Secondly, a screen can only identify the first essential function of a gene. Imagine a screen for eye development. A gene involved in eye development will not be identified if it is essential for initial embryogenesis as no embryos will survive to show a defect in eye development. Finally, some genes are redundant, either by virtue of gene duplication or by the action of two pathways ultimately controlling the same aspect of phenotype.

There are several ways around the problem of only being able to observe the first essential function of a gene. Enhancer screens can be important here: weak mutations can be isolated in genes where the mutation elicits the phenotype of interest because it is sensitised, but other phenotypes are not elicited because the mutation is not strong enough. Also important are conditional mutants as touched upon earlier in the context of temperature-

sensitive mutants in yeast. In other organisms mutations can be made conditional upon tissue type in so-called clonal screens. Here, meiotic recombination is induced after embryogenesis producing clones of homozygous mutant cells in otherwise heterozygous mutant individuals. This can be induced using X-rays or the Flp/FRT site-specific recombination system from the yeast 2 μ plasmid. Flp/FRT can be heat inducible or made tissue specific by putting the Flp recombinase under the control of a tissue-specific promoter. Xu *et al* used this system to screen for genes which lead to uncontrolled proliferation in the imaginal discs and identified several candidate tumour suppressor genes (Xu et al. 1995).

1.1.1.5 Forward genetic screens in cell culture

Screening in tissue culture cells (the only sort of screening that can be performed in human systems) by random mutagenesis is hampered by the difficulty of generating the homozygotes necessary for screening for recessive mutants, and the lack of recombination makes identifying the causative gene difficult. Ting *et al* used a selection to isolate rare loss-of-function mutants that no longer activated NF- κ B in response to TNF. Comparison of the mutant and parental lines showed that a candidate (RIP) was not expressed (Ting, Pimentel-Muinos & Seed 1996). More often, screening in cell culture involves over-expressing genes by introduction of cDNA libraries, a procedure known as expression cloning. cDNA libraries are introduced into cells. Clones showing the phenotype of interest are selected, the plasmids recovered and amplified in bacteria and retransformed into cells, enriching for the plasmid of interest. Alternatively, defined pools of cDNA clones are introduced into cells, which are then screened for the phenotype of interest. Positive pools are sub-divided and re-transfected. Multiple rounds of this can lead to pools small enough to allow the testing of single cDNA clones and the identification of the causative clone. Many mammalian genes have been isolated this way, including the transcription factor GATA1, where clones were tested for their ability to bind a certain DNA sequence (Tsai et al. 1989).

1.1.1.6 Reverse Genetics

The availability of genome sequences and gene predictions for most model organisms as well humans has opened the way for a move from forward genetics to reverse genetics. In reverse genetics rather than starting with the phenotype and working back to the gene via a mutation, the starting point is the gene, which is specially mutated and the effects studied. In yeast collections of strains exist with null mutation in more than 90% of ORFs. A similar project – the knock-out mouse project (KOMP) - aims to generate null mutations, tagged

with a suitable selectable marker in a large proportion of all mouse genes. Here, each allele is sequentially targeted with a disruption cassette. In other species collections of mutants in all genes are being assembled as they are generated. For example, the Bloomington stock centre now holds mutations in genes representing a large portion of genes in the *D. melanogaster* genome (Bellen et al. 2004). Genes of known sequence can also be targeted using RNA interference (see below). Collections of resources for over-expression also exist. For example the ORFeome collections in *Schizosaccharomyces pombe*, worms and humans aim to make available one expression clone representing each ORF in that organism (Matsuyama et al. 2006)

Reverse genetic screens involve taking a large or genome-wide collection of knock-out/overexpressing clones and screening them for the phenotype of interest. One interesting application is Synthetic Genomic Array (SGA) screens. Here yeast strains representing each of the yeast's non-essential genes are mated to systematically produce double mutants, which carry knock-outs in two genes. The study of these knock-outs allows for the examination of the interaction between the two genes and the discovery of synthetic phenotypes. A synthetic phenotype is one which is visible in a double mutant when both single mutants are normal. This has been used to systematically assay for synthetic effects of viability (Tong et al. 2001). Selections can also be used in reverse genetics. Here, a large pool of defined mutants is cultured together and a selection applied, with surviving clones isolated and the responsible gene determined, or the pool deconvolved. An application of this in yeast involved using a unique oligonucleotide sequence included with the deletion – a molecular barcode. Yeast were grown competitively for several generations in both rich and minimal media. DNA from the resulting mix of strains was isolated and a microarray was used to determine which barcodes were over- or under-represented, identifying genes which had an effect on fitness (Winzeler et al. 1999).

The advantage of genome-wide reverse genetic screening is that, in theory, every gene is tested and reaching saturation is not an issue. It should, in theory, also be efficient as only one mutation per gene is necessary, whereas in forward genetics, in order to ensure one mutation in most genes, far more mutants than genes must be generated.

1.2 RNA interference

The ability of exogenously introduced double-stranded RNA to induce a reduction in the activity of gene products – RNA interference (RNAi) – is one of a growing collection of small RNA mediated gene regulatory mechanisms, including the micro RNA pathway, the

piRNA pathway and the rasiRNA pathway. Initially observed when attempts to use sense RNA as a control for antisense-mediated knock-down resulted in a similar phenotype to that of antisense RNA in the *C. elegans* (Guo, Kemphues 1995), it was first rigorously described in the worm in 1998 by Fire and colleagues (Fire et al. 1998). RNAi mechanisms function in a wide range of organisms, including flies (Clemens et al. 2000), Zebrafish (Li et al. 2000), mouse embryos (Svoboda et al. 2000) and mammalian tissue culture (Elbashir et al. 2001). In plants, the silencing effect of double-stranded RNA is known as post-transcriptional gene silencing (PTGS) or co-suppression (Napoli, Lemieux & Jorgensen 1990). RNAi is triggered when small 21-22nt double-stranded RNAs (siRNAs) are incorporated into a multi-protein complex known as the RNA-Induced Silencing Complex (RISC) where they guide the cleavage of complementary mRNAs (Schwarz et al. 2002).

This approach has proved very useful in single-gene analysis, especially in mammalian cell culture, where other methods for reducing the function of particular genes are not as quick, easy or as effective. The use of RNAi as a therapeutic agent is also being rigorously investigated by many. However, the one very exciting prospect is the use of RNAi as a screening tool. Screening in this way is now well established in *C. elegans* and *Drosophila* and is becoming more common in mammalian cell culture. Genome-scale RNAi screening in mammalian cell culture is particularly exciting owing to the lack of other methods for generating such large numbers of hypomorphs in such a short time.

As with any technique, RNAi is not perfect. There are three main problems. Firstly, many of the methods generate only a transient knock-down of gene activity, although RNAi in the worm is heritable, at least for a number of generations, and knock-downs can be made heritable in other systems by expressing double-stranded RNA or hairpin constructs from transgenes. The second is that gene knock-down is not gene knock-out and the use of different RNAi-inducing reagents against different genes results in different levels of residual mRNA. This has the advantage that levels of essential genes can be reduced without being eliminated, and in some cases the construction of a series of knock-downs of different efficiencies can be useful (Hemann et al. 2003). However, it also means that a given RNAi-inducing agent may not knockdown the targeted mRNA sufficiently to elicit a phenotype. Finally, questions surround the specificity of RNAi knock-downs, particularly those induced using chemically synthesised siRNAs or hairpins in mammalian cell culture. Several reports have demonstrated unintended consequences of gene knock-down which can be dependent on the sequence of the siRNA/shRNA in question (Birmingham et al. 2006, Jackson et al. 2003, Lin et al. 2005, Lin et al. 2007) or independent of the sequence (Bridge et al. 2003, Kim

et al. 2004, Pebernard, Iggo 2004, Sledz et al. 2003).

1.2.1 Triggers of RNAi

There are a number of ways that RNAi can be triggered. In general, RNAi can be triggered by long dsRNA in non-mammalian model organisms and in mouse stem cells. In the worm, this dsRNA can be introduced by either injection, soaking worms in a solution of dsRNA, or feeding worms bacteria expressing the dsRNA (Fire et al. 1998, Maeda et al. 2001, Timmons, Fire 1998). A library of bacterial clones which can inducibly express dsRNA targeting each of the predicted genes in the *C. elegans* genome was constructed quickly after the discovery of RNAi in the worm (Fraser et al. 2000, Kamath et al. 2003). These clones can either be fed directly to worms or can be used to prepare dsRNA that can be used for soaking or micro-injection.

RNAi can also be induced in *D. melanogaster* by micro-injection into embryos (Kennerdell, Carthew 1998). However, a far more common use of RNAi in *Drosophila* genetics is knock-down of genes in cell culture. In some cases, dsRNA is taken up by the cells directly from the medium (Clemens et al. 2000), in others it is necessary to transfect the dsRNA (Lum et al. 2003). A number of large/genome-wide collections of dsRNA expressing clones are available for *Drosophila* genes (Reviewed in Echeverri, Perrimon 2006).

In mammalian cell culture, RNAi is induced by short dsRNAs known as siRNAs. These siRNA are 21bp long and have 2nt 3' overhangs, and either 5' terminal hydroxyl or phosphate groups. They can be introduced either by transfection of oligonucleotides (Elbashir et al. 2001)(Figure 1.1a) or by transcription of short hairpin structures, known as shRNAs, from plasmids introduced into the cell either by transfection or viral infection (Paddison et al. 2002). shRNAs are processed by the cellular RNA silencing machinery to produce siRNAs (see section 1.2.2). shRNAs come in two varieties: Type I shRNAs consist of the sense and anti-sense sequences separated by a short loop sequence(Paddison et al. 2002)(Figure 1.1b). Type II shRNAs are modelled on miRNAs and contain miRNA flanking and loop sequences (Silva et al. 2005)(Figure 1.1c). siRNAs can either be chemically synthesised or transcribed *in vitro* or *in vivo* (Zheng et al. 2004)(Figure 1.1d). Finally, a slightly different approach, known as endoribonuclease-prepared siRNA (esiRNA) involves transcribing long dsRNAs *in vitro* and using recombinant Dicer or RNaseIII to digest the long dsRNA into short siRNAs. This produces a pool of siRNAs, all targeting the same gene (Kittler et al. 2004)(Figure 1.1e).

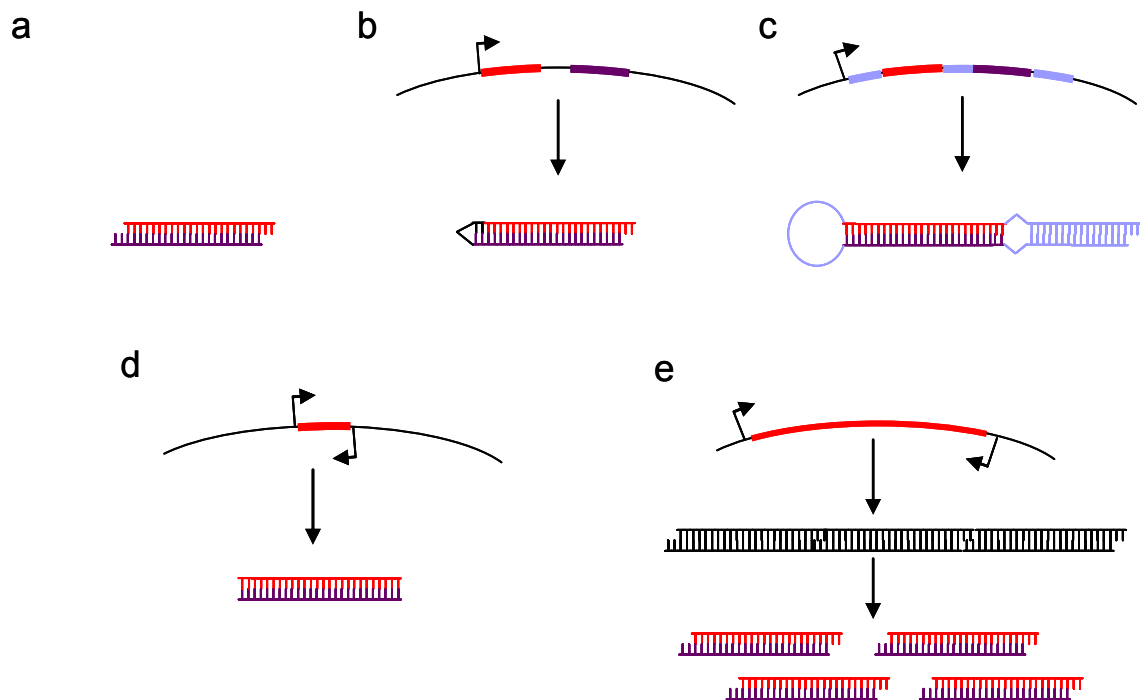


Figure 1.1 RNAi triggers used in mammalian cell culture.

Red – anti-sense strand (complementary to target), Purple – sense strand (identical to target), blue – miRNA sequence. a) Chemically synthesised siRNA, b) Type I shRNA, c) Type II shRNA, d) Transcribed siRNA, e) esiRNA.

There are a number of readily available libraries of RNAi-inducing reagents targeting human genes. Libraries of siRNAs targeting all or most mammalian genes are sold by several companies (including but not limited to Ambion, Dharmacon and Qiagen). These libraries contain between two and four siRNAs targeting each gene, with each siRNA either in a separate well (Ambion, Qiagen) or pooled according to gene (Dharmacon). The effect of pooling is disputed. Those that pool their siRNAs claim that it increases the potency compared with the average of the individual siRNAs contained within the pool and that, importantly, it also reduces off-target effects (see section 1.2.3) by diluting the off-target effect of each individual siRNA. However, those that do not pool claim that a pool is not as effective as the best siRNA in it and that one “dirty” siRNA can increase the off-target effects of the whole pool. It would, however, be important to ensure that in a siRNA pool each siRNA is incorporated into RISC with a similar efficiency to prevent competition.

At the time of starting this project, two large shRNA libraries were available (Berns et al. 2004, Silva et al. 2005). These libraries differ in several characteristics, summarised in Table 1-1. The most important differences are the type of hairpin (Type I vs. Type II), the inclusion of barcodes in the Silva library, and the fact that the Silva library is sequence verified (although the Berns library is being sequenced).

Feature	Silva et al	Berns et al
Number of clones (2004/present)	18,882/81,500	23,742/23,742
Number of genes (2004/present)	13,456/28,500	7,914/7,914
Vector	pSHAG-MAGIC2	pRetroSuper
Vector introduction methods	Transient Transfection Stable Transfection Viral Infection	Transient Transfection Stable Transfection Viral Infection
Barcodes	Separate 60bp barcodes	Uses hairpin sequence
Promoter	U6	H1
Other vector features	MAGIC cloning system	
shRNA type	Type II	Type I
shRNA cassette design	Proprietary, rule based Based on miRNAs Three mismatches to any other gene	Based on 9 rules including: 19bp in length Start with C/G (to introduce strand bias) Share minimal homology with other targets 30%-70% CG
Redundancy	Variable: Some genes have only one hairpin, some have up to 9.	Three hairpins per gene
Pooling	None	Available as per gene, per plate and per library
Sequence verification	Yes	No

Table 1-1 Comparison of the shRNA library available at start of project

1.2.2 Mechanism

Biochemical and genetic analysis has outlined the mechanism through which double-stranded RNA silences gene products. A summary of the current model is presented in Figure 1.2.

RNA interference is triggered by short double-stranded RNAs (Elbashir, Lendeckel & Tuschl 2001) known as siRNAs. As well as being 19bp-22bp in length, siRNAs have several other features. Important other structural features include having characteristic 2nt 3' overhangs and phosphorylated 5' ends. They come from a range of sources including exogenous long double-stranded RNA (dsRNA) and short hairpin structures (shRNAs). Long dsRNAs are converted into siRNAs by Dicer endonucleases, which are multidomain ribonuclease III enzymes (Bernstein et al. 2001). shRNAs originate either from natural long primary transcripts known as pri-miRNAs, which are then processed to form the short-hairpin pre-miRNAs (Lee 2002), or from artificial constructs expressed from plasmid or viral vectors (Paddison et al. 2002). The first step in the processing of pre-miRNAs or constructs designed to mimic them is enacted by the Microprocessor complex, which contains the

RNase III enzyme Droscha and its dsRNA-binding protein (dsRBP) partner DGCR8 (known as Pasha in *Drosophila*) (Denli et al. 2004). Pre-miRNAs are formed in the nucleus and then exported into the cytoplasm by exportin-5, which also exports artificial shRNAs (Yi et al. 2003). Pre-miRNAs and artificial shRNAs are converted to siRNAs through the action of Dicer (Hutvagner et al. 2001, Paddison et al. 2002). In *Drosophila* miRNA processing is carried out by Dicer-1 and dsRNA processing by Dicer-2, suggesting distinct pathways for siRNAs and miRNAs (Lee et al. 2004 Apr 2). However, mammals and worms have only one Dicer protein.

The antisense strand, or guide strand, of the siRNA guides a multiprotein complex known as the RNA Induced Silencing Complex (RISC) to the target mRNA to which the guide strand is complementary. If the siRNA guide strand is completely complementary, RISC then cleaves the target at a position opposite the bond between the 10th and 11th nucleotides of the guide (Elbashir et al. 2001). The endonuclease at the heart of RISC has been shown to be Argonaute-2 (Liu et al. 2004, Rand et al. 2004). Argonaute proteins have various roles in small RNA-silencing pathways, but in humans only Argonaute-2 is capable of endonuclease activity ((Reviewed in Peters, Meister 2007).

Although purified Argonaute-2 can bind single-stranded RNA to reconstitute RISC activity, it is not able to load double-stranded siRNA (Rand et al. 2004). The loading of siRNA into RISC goes through two intermediates known as Complex B and the RISC Loading Complex (RLC, otherwise known as Complex A) (Tomari et al. 2004a). In *Drosophila*, the RLC contains at least Dicer-2 and the dsRBP R2D2 (RNA binding domains x 2 associated with Dicer-2). R2D2 binds to the phosphorylated 5' strand at the most stable end of the siRNA duplex, simultaneously specifying which strand will be loaded into RISC (not the one it is bound to) and licensing the siRNA for entry into RISC (Tomari et al. 2004b). This provides a mechanism for the observed asymmetry, whereby the strand of the siRNA which has a lower thermodynamic stability at its 5' end is preferentially loaded into RISC (Schwarz et al. 2003). In mammals it has been suggested that the dsRBPs TRBP and PACT play this role. They contain multiple RNA-binding domains homologous to R2D2 and are found in complexes with Dicer and Argonaute-2 (Chendrimada et al. 2005, Lee et al. 2006). However, the precise role of these dsRBPs in the RNAi pathway is unclear. Some have reported that they are required for siRNA assembly into RISC (Chendrimada et al. 2005, Gregory et al. 2005, Lee et al. 2006). Chendrimada *et al* demonstrated that knock-down of TRBP reduces miRNA processing, but that this was likely due to a destabilisation of Dicer (Chendrimada et al. 2005). In contrast, it has also been reported that both TRBP and PACT are dispensable

for siRNA mediated silencing, but are required for miRNA processing (Kin et al. 2007).

There are two models for the mechanism by which active RISC, bound to the guide strand of the siRNA, is formed from Dicer-TRBP-PACT, double-stranded siRNA and Argonaute-2. It was originally believed that the double-stranded siRNA was unwound by a helicase, with the guide strand retained, which was then progressively inserted into RISC. This was based on the finding that Argonaute was only found associated with single-stranded RNA and that RISC assembly was ATP dependent (Nykanen, Haley & Zamore 2001). A role in RISC formation has been shown for several helicases (Meister et al. 2005, Robb, Rana 2007, Tomari et al. 2004a).

However, recently a second model for the loading of siRNA into RISC has emerged. It has been demonstrated that in fact Argonaute-2 can be found associated with double-stranded siRNA and that it cleaves the passenger strand, facilitating its dissociation from RISC, and that this process is ATP independent (Matranga et al. 2005, Rand et al. 2005). It is not clear that these two models are mutually exclusive, since imperfectly matched miRNAs are not cleaved by Argonaute-2, and in humans, siRNAs and miRNAs associate with all four Argonaute proteins, but only Argonaute-2 has been demonstrated to have endonuclease activity.

In nematodes, plants and fungi, the primary siRNAs derived from long dsRNA trigger the production of secondary siRNAs outside the region covered by the original dsRNA, a process known as transitive RNAi. This process is dependent on the action of RNA dependent RNA polymerases (RdRPs). In *C. elegans* this activity is encoded by the RRF-1 gene (Sijen et al. 2001). These secondary siRNAs form a separate class of RNAs which are biochemically distinct, function through separate Argonaute proteins, and do not themselves trigger amplification (Pak, Fire 2007). RNAi in *Drosophila* and mammals is not dependent on RdRPs (Schwarz et al. 2002) and no evidence of transitive RNAi has been found for these species. In plants and some yeast (such as *Schizosaccharomyces pombe*), siRNAs can act to silence transcription by triggering methylation of DNA. There is some evidence that siRNAs targeted at promoters, but not coding sequences, can direct methylation-dependent transcription silencing in mammals (Kawasaki, Taira 2004, Morris et al. 2004, Park et al. 2004). This has been shown to be dependent on Argonaute-1 (Kim et al. 2006).

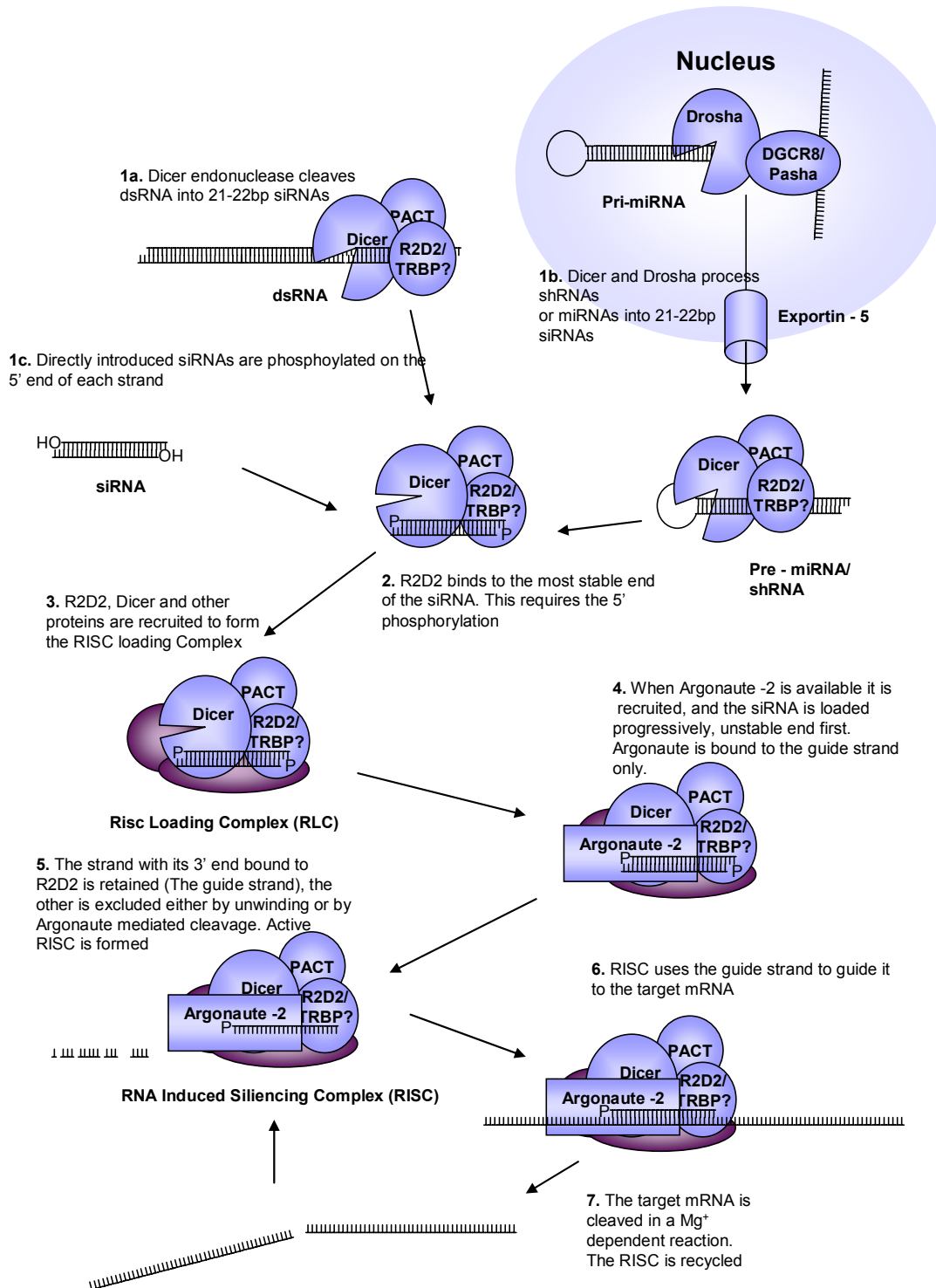


Figure 1.2 A possible model for siRNA mediated mRNA degradation

1.2.3 Design of potent RNAi inducing agents

Not all siRNAs are of equal potency in knocking down target mRNAs (Elbashir et al.

2001). The original guide-lines for the design of siRNAs specified few factors for increasing the sequence dependent potency of the siRNA. These included that the siRNAs should be 21bps in length, with 2nt overhangs, which should be UU or UG (or dTdT/dTdG) on both strands and the duplex should have a low GC content (Elbashir et al. 2001, Elbashir et al. 2002).

Subsequently much work has been carried out to determine what makes a potent siRNA. Some factors, such as that siRNAs should show a lower T_m at the 5' end of the guide strand were deduced from studies of the biochemistry of the RNAi pathway (Schwarz et al. 2003). Others have been deduced from analysis of the potency of a large number of randomly chosen siRNAs. Properties predicted include a lack of inverted repeat sequences, a lack of tracts of 9nt or more comprising entirely G or C and a variety position specific base preferences (Reynolds et al. 2004, Ui-Tei et al. 2004). Such determinants have been extensively reviewed (for example Patzel 2007)

The finds of these studies are drawn together in the many different algorithms for designing siRNAs. These can be based either rule based systems (Holen 2006) or use artificial intelligence methods such as neural networks (Huesken et al. 2005) or support vector machines (Jia et al. 2006). Many of these algorithms have been made available as design tools both by academic and commercial groups (Patzel 2007)

1.2.4 Specificity

One of the most important factors when considering the usefulness of RNA interference as a tool for large-scale screening, the confidence with which results can be regarded, is the specificity of gene knock-down. Originally, RNAi was reported to be very specific (Elbashir et al. 2001, Elbashir et al. 2001). However, it is now understood that there can be a range of unintended effects that accompany the knock-down of the targeted gene.

A discussion of the so called “off-target” effects of an siRNA or shRNA can be divided between those effects which are dependent on the sequence of the RNAi-inducing molecule and those effects which are independent of sequence. These can be distinguished by studying the effects of si/shRNAs targeting the same gene. Consider the hypothetical experiment presented in Figure 1.3. In Figure 1.3a, siRNAs targeting the same gene have the same effect, and that effect is different from the effect of siRNA targeting another gene. Note that in this situation, transcripts other than the one targeted may be affected by events downstream of silencing the targeted transcript. In Figure 1.3b, transcripts other than the targeted transcript are affected and the effects are different for different siRNAs targeting the

same gene – that is the effects are sequence dependent. Finally, in Figure 1.3c transcripts other than the targeted transcript are affected, but all siRNAs have the same effect, even though they are targeting separate genes, that is, they are sequence independent.

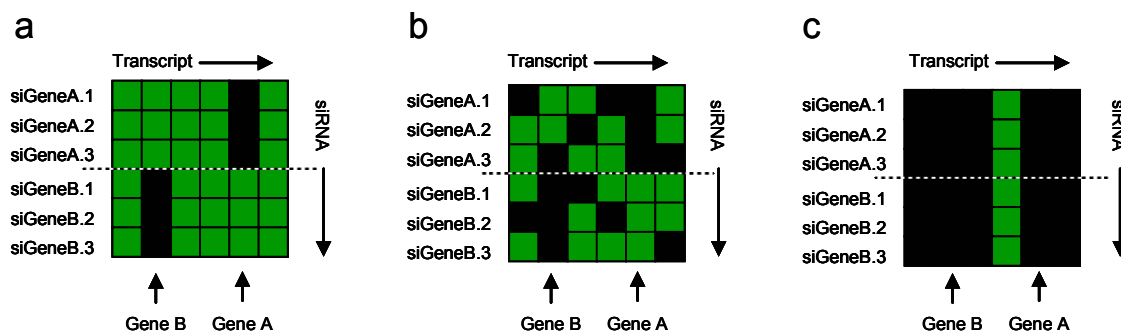


Figure 1.3 Sequence specific and sequence independent effects of siRNAs.

A hypothetical microarray experiment. Six siRNAs, targeting two genes are transfected into six populations of cells. mRNA is harvested and analysed on microarrays. Black cells represent genes that under-expressed compared with green cells a) On-target effects. Each siRNA regulates the expression of the same transcript as the other siRNAs targeting that transcript. b) Sequence specific off-target effects. Expression of transcripts other than the transcript targeted are changed and different siRNAs targeting the same gene have differing effects. c) Sequence independent off-target effects. Expression of transcripts other than the transcript targeted are changed and siRNAs targeting different genes have the same effect. After (Jackson, Linsley 2004).

1.2.4.1 Sequence specific off-target effects

Original reports of RNAi in mammalian cells using siRNAs found high levels of specificity, with as little as a one base mismatch resulting in a drastic reduction in knock-down efficiency (Elbashir et al. 2001). However, it has been shown that the RNAi machinery is mostly tolerant to single-base mismatches, although the degree of tolerance is dependent on the position and identity of the mismatched bases (Du et al. 2005).

Initially, microarray experiments surveying the effect of siRNA transfection on a transcriptome level supported the idea that RNAi was specific (Chi et al. 2003, Semizarov et al. 2003), finding either that knock-down affected only the targeted gene (Chi et al. 2003), or that off-targets could be eliminated by reducing the concentration of siRNA or by more restrictive design (Semizarov et al. 2003). However, another report in the same year found large numbers of transcripts were affected by transfection of siRNAs and that only a small number were in common between different siRNAs targeting the same gene (Jackson et al. 2003). It was found that off-target effects fell into one of two groups. One group consisted of transcripts that shared a region of homology to the central 14 bases of the siRNAs. The second group consisted of transcripts that had much shorter regions of homology, as little as 7 bases, to the 5' end of one strand of the siRNA.

This was supported by a larger, more systematic study into the relationship between

siRNA sequence and the targets silenced. Birmingham *et al* created a large database of off-target effects elicited by a number of siRNAs targeting a number of genes. They found that overall sequence identity below 1 or 2 mismatched nucleotides was not correlated with knock-down. However the off-target effects were associated with the presence of one or more 6 or 7 base “seed” sequences homologous to nucleotides 2-7 or 2-8 of the siRNA guide strand in the 3' UTR of transcripts (Figure 1.4). The more of these seed sequences present, the greater the probability that the gene would be affected. However, not all transcripts containing seed sequences in their 3' UTR were affected, indicating that other determinants are important (Birmingham *et al.* 2006).

Lim *et al*, also found that 7nt complementary in the 5' seed region of the siRNA was sufficient to cause off-target knock-down of transcripts. They conducted a screen for regulators of the HIF-1 pathway. They found that two of their top three hit siRNA shared the same 7nt sequence in the seed region and that this was complementary to two sequences in the 3' UTR of HIF-1 α . They also found that the context of the 7nt match was important (Lin *et al.* 2005).

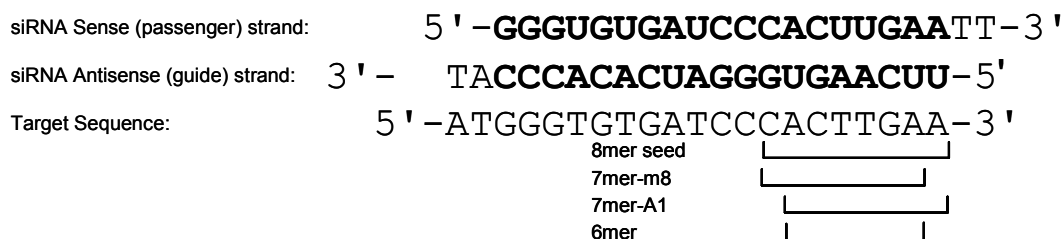


Figure 1.4 Different types of seed sequence as defined by (Lewis, Burge & Bartel 2005)

The importance of 6nt-7nt seed sequences in the 5' region of the siRNA guide strand is reminiscent of the 6nt-7nt seed sequence that has been found to be important in the specificity of micro RNAs (Brennecke *et al.* 2005, Doench, Sharp 2004). Several lines of evidence support the hypothesis that at least some off-target effects are due to siRNAs acting as miRNAs. Firstly, siRNAs, like miRNAs, may regulate genes with which they are mismatched at the transcript level or protein level (Bagga *et al.* 2005, Wu, Fan & Belasco 2006). There are several reports of siRNAs affecting protein levels of genes but not affecting transcript levels to the same extent. (Alemán, Doench & Sharp 2007, Saxena, Jonsson & Dutta 2003) . So-called GU wobble base pairing between the siRNA and the mRNA has been shown to reduce mRNA silencing, but not protein silencing (Alemán, Doench & Sharp 2007, Saxena, Jonsson & Dutta 2003) . Secondly, it has been shown that degradation of off-target mRNA is less well correlated with cleavage at the canonical siRNA cleavage site, and

may be independent of Ago2, which is the endonuclease involved in RNAi mediated silencing (Alemán, Doench & Sharp 2007) . miRNAs associate with all four human Argonaute proteins, but only Ago2 is capable of specific cleavage of the target mRNA. miRNA associated mRNA degradation is thought to occur at least partly via the recruitment of decapping and de-adenylation proteins causing a destabilisation of the message (Behm-Ansmant et al. 2006, Wu, Fan & Belasco 2006)

The 3' UTRs of genes targeted by miRNAs often contain multiple seed sequences for multiple miRNAs. It has been shown that the optimal spacing for these seeds is 13nt-26nt. Seeds that are closer than 13nt actively interfere with one another, and any cooperative effect from having multiple seeds in a UTR is dramatically reduced for seeds spaced further than 26nt away from one another (Grimson et al. 2007, Sætrom et al. 2007) . Sætrom *et al* also examined the location of matches to the seed sequences of the siRNAs used in Birmingham *et al* in the 3'UTRs of genes which were shown to be regulated by off-target effects. They found that there was an under-representation of matches to the seed sequences of miRNAs known to be expressed in the cell type used by Birmingham *et al* within 13nt of the matches to the seed sequences of siRNAs used in the study. They also found an over-representation of matches to the seeds of these siRNAs within 26nt of seed sequences for co-expressed miRNAs in the 3'UTRs of transcripts shown to be regulated by these siRNAs. This suggests that the effect of siRNAs is dependent on co-targeting of transcripts by miRNAs and as such would mean that off-target effects would be cell-type specific to a certain extent.

There is now convincing evidence that siRNAs can affect the expression of untargeted genes at both the mRNA and protein level. It is possible that these effects are mediated in two ways. Firstly, the RNAi machinery would seem to be less sensitive to single or double mismatches in otherwise perfectly matching targets. Secondly, some of these effects are due to siRNAs acting in the miRNA pathway. As miRNA target-site prediction improves, it might be possible to predict off-target effects (Grimson et al. 2007), but this is not currently feasible. It has also been reported that chemical modification of siRNAs can increase their specificity. Addition of 2' O-methyl groups to bases of the passenger strand reduces its incorporation into RISC, therefore reducing any off-target effects due to this strand. Addition of 2' O-methyl groups to base 2 of the guide strand can also reduce, but not eliminate, off-target effects, from this strand. However, off-targeted genes with perfect matches to the seed region are less affected.(Jackson et al. 2006). The mechanism of this effect is not known. The authors of the original report speculate that the addition of the modification to bases in the seed region reduces the stability of pairings between the siRNA

and the target mRNA in this region, meaning longer matches are required for proper pairing. Alternatively, they also suggest that possibly the modification renders RISC incapable of cleaving non-perfectly matched targets (Jackson et al. 2006). However, if off-target effects are due to miRNA like effects, as is suggested by multiple lines of evidence explored above, then this second explanation would seem unlikely as degradation of miRNA targets is probably not due to cleavage by RISC (Alemán, Doench & Sharp 2007) . In order to control for possible off-target effects it is important that all RNAi experiments are confirmed by at least 2 independent siRNAs/shRNAs. This is particularly important in screening applications, as hits are likely to be enriched in siRNAs/shRNAs that have off-target effects on genes involved in the process under study.

One interesting possibility is that this knowledge of off-target effects could be used to extract more information from screening datasets. One group has reported that several hits identified in a screen could be ascribed to off-target effects. They took the seed sequences of siRNAs which were causing off-target effects and used them to search for matches to a database of 3' UTRs. In this way they were able to find genes that could be regulated by the off-target effects of these siRNAs. In this way they identified a gene which was regulated by several of siRNAs causing off-target hits and which was important in the process they were studying, but which had been missed by their primary screen. (Lin et al. 2007).

1.2.4.2 Sequence-independent off-target effects

It was initially thought that RNAi in mammalian cells would not prove possible because double-stranded RNA triggered a general anti-viral response termed the interferon response in such cells. This response includes a general shut-down of translation and non-specific degradation of mRNA. It was originally thought that double-stranded sequences of a length shorter than 30 bp could escape this response (Manche et al. 1992). However, there are now several reports of induction of interferon-response-related genes in a non-specific manner after the introduction of RNAi-inducing molecules.

One study used microarrays to demonstrate that up to 50 interferon-response related genes were up regulated when siRNAs targeting several genes were transfected into human cells. Mouse cells with a knock-out in the gene responsible for recognising dsRNA and activating the interferon response, PKR, did not exhibit this response (Sledz et al. 2003). The mechanism of this induction remains unknown, although it is known that blunt-ended siRNAs can trigger the interferon response via an interaction with RIG-1 and that siRNA

purity can affect interferon induction (Marques et al. 2006). The response has also been shown to be cell-type and transfection-reagent specific (Yoo et al. 2006). Others have found that shRNAs expressed from plasmid vectors using the U6 or H1 promoters can induce an interferon response (Bridge et al. 2003) and that in the case of the U6 promoter this was due to the presence of an AA motif in the promoter sequence (Pebernard, Iggo 2004). Yet another possible trigger of the interferon response is the triphosphate found on the end of siRNAs produced by *in vitro* transcription with T7 RNA polymerase (Kim et al. 2004). Although in none of these cases do the authors examine the effects of the interferon response on the viability of the transfected/infected cells, it can be implied that there is no massive effect on viability at the time points the investigators examined. Most of the reports involve measuring the transcript levels of genes involved in the response either by microarray or by real-time PCR, therefore, viable cells must be present to allow the isolation of RNA. This is despite in some cases a report induction of interferon response markers of up to 500 fold (Bridge et al. 2003).

It has been found that certain GU-rich sequence motifs in siRNAs can trigger an inflammatory immune response in some cell types, possibly through the activation of the Tol receptor pathway (Judge et al. 2005). Although this is technically a sequence specific reaction, it is included here as it is a reaction to the siRNA itself rather than a consequence of an off-target RNAi effect.

A separate way in which siRNAs/shRNAs can trigger a non-specific response is by the overloading of the endogenous RNAi/miRNA machinery. In theory, one could imagine that saturating the RNAi/miRNA machinery with siRNA or shRNA might lead to a reduction in the activity of endogenous miRNA. Thus far there have been no reports of this for siRNAs. However, shRNAs have been reported to cause toxicity in the livers of mice after virus-mediated delivery. This effect was accompanied by a reduction in miRNA levels and miRNA mediated silencing. This competition could be relieved by the over expression of exportin-5, a protein believed to be involved in the transport of pre-miRNAs and shRNAs from the nucleus into the cytoplasm (Grimm et al. 2006).

Although it is possible to eliminate some of these general responses using careful siRNA/shRNA and vector design, this still cannot guarantee a complete lack of response. All of the effects reported here are concentration dependent, so it is important to use the lowest concentration of siRNA/shRNA that elicits the intended response. Further, it also emphasises the importance of comparing the effect of siRNA silencing with non-targeting controls as well as non-transfected controls. Ideally, induction of the interferon response

could be controlled for by measuring the induction of interferon response associated genes, such as OAS1 by quantitative reverse-transcription PCR (qRT-PCR).

1.2.5 RNAi Screening

1.2.5.1 Screening Paradigms

There are a multitude of ways in which an RNAi screen can be performed depending on the organism, the process being studied, and the library being used. Several different paradigms are summarised in Figure 1.5.

The most obvious paradigms, and the ones most widely applicable, are the gene-by-gene approaches, where the screen consists of a large number of experiments, usually carried out in a multi-well plate, where the output is either some sort of bulk measurement, usually fluorescent or luminescence based, read by a plate reader, or recorded down a microscope. Bulk outputs provide the quickest, easiest, and possibly most quantitative outputs, but microscopy can provide a higher density of information including the recording of multiple phenotypes. Microscopy also allows for the examination of a wider range of phenotypes including unexpected ones. However, the quantitative recording of phenotypic information from a large number of RNAi knock-downs requires either a large number of man-hours, or highly specialised automated microscopy platforms and image-analysis pipelines.

One way in which the throughput of high-content screening can be increased in cell culture screens is by using RNAi arrays. Here, the RNAi-inducing agents are mixed with transfection reagent and spotted on to known locations on a glass slide, along with some sort of transfection marker. Cells are then grown as a monolayer on the surface of the slide. The slide is scanned using a slide-imaging platform. Transfected cells are marked and their phenotype recorded, the gene knocked-down being determined by the position on the slide.

Pooling RNAi-inducing agents and performing selections rather than screens has the advantage of greatly increasing the throughput of a study, allowing the entire genome to be examined in 100-1000 fold fewer individual experiments than gene-by-gene approaches. A collection of RNAi-inducing agents targeting a number of genes is introduced into cells as a pool and a selection is applied such that cells showing the phenotype of interest are separated from the majority of cells. The phenotype-causing RNAi-inducing agent is then identified. Here, the use of shRNAs is particularly applicable, as the knock-down of interest can be determined by examining the hairpins present in the selected clones. This can be done by the identification of either hairpins sequences themselves or the identification of associated

barcodes. Identification can either be by sequencing or hybridisation to a microarray of hairpin/barcode sequences. Unless the selection employed has a very low escape rate and some method of ensuring only one shRNA is introduced into each cell, it is the enrichment of hairpins that is important rather than just the presence of hairpins. Here, barcode arrays probably have the edge unless a method of sequencing many hairpins is employed. One possibility would be to extract hairpin/barcode sequences and ligate several together to form a concatemer, such that a single sequencing reaction could cover several hairpins/barcodes. Although such avselection based screen would greatly increase the throughput of a study, it would restrict the processes that can be studied to those that allow selection, such as survival assays or assays where outcomes can be separated by fluorescence activated cell sorting (FACS). Also, the assay must allow few negative clones to escape.

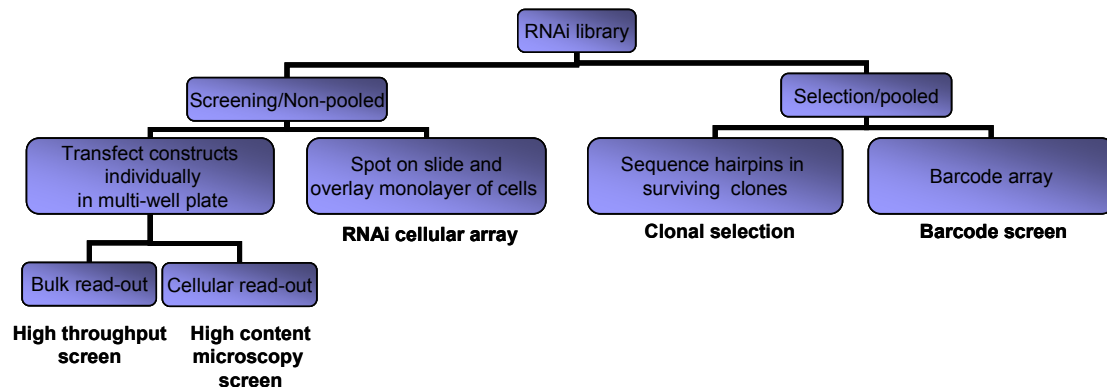


Figure 1.5 Paradigms for genome-scale RNAi screening

1.2.5.2 Screening in model organisms

The first RNAi screens in model organisms, indeed the first RNAi screens in any organism, were screens of all predicted genes on particular chromosomes of the worm *C. elegans* (Fraser et al. 2000, Gonczy et al. 2000). Gönczy *et al* micro-injected dsRNAs in pairs, covering most of the genes on chromosome III, and screened for defects in embryogenesis using time-lapse microscopy. Pairs of dsRNAs that elicited a phenotype were tested separately. In this way they identified 133 genes necessary for a range of cellular processes in embryogenesis. In contrast, Fraser *et al* preferred to assay for a wider range of phenotypes. They fed worms on bacteria expressing dsRNA homologous to predicted genes on chromosome I. They assayed the offspring of these worms for obvious embryonic and post-embryonic phenotypes. In this way, they assigned functions to 13.9% of the genes they examined, noting that knock-down of conserved genes was more likely to elicit a phenotype than knock-down of non-conserved genes.

Soon after, the first genome-wide survey of gene function in the worm was published

(Kamath et al. 2003). The screen was carried out in a similar fashion to Fraser *et al.* Worms were fed bacteria expressing dsRNA corresponding to 86% of the worm genome. Information on sterility, embryonic or larval lethality, slow growth and a wide range of post-embryonic phenotypes was recorded from the offspring of fed worms. Phenotypes were recorded for 10% of genes, including 77.2% of genes whose mutation is known to cause a non-viable phenotype and 43% of genes whose mutation is known to cause a post-embryonic phenotype.

Many screens for more focused phenotypes have now been conducted, including screens for genes involved in fat regulation (Ashrafi et al. 2003), mutator genes (Pothof et al. 2003), genes involved in poly-glutamine aggregation (Nollen et al. 2004), genes involved in the RNAi pathway (Kim et al. 2005), genes involved in axon guidance (Schmitz, Kinge & Hutter 2007), genes involved in the non-sense mediated mRNA decay pathway (Longman et al. 2007) and many more.

C. elegans presents an excellent model for studying the organisation and development of multicellular organisms and the malfunction of such in disease. However, direct study of biochemical processes underlying cell biology is often best undertaken in cell-culture systems where the output is measurement of some surrogate for the biochemical state of the cell. Knock-down of a gene may have no obvious effect on the organism as a whole, and yet may profoundly change the state of the cell. Among the model organisms, *D. melanogaster* is the system most widely used. The first RNAi screen in the *Drosophila* cell culture involved the knock-down of 1,000 randomly selected genes taken from a cDNA library. The effect of these knock-downs on the phagocytotic abilities of S2 cells was examined, identifying a receptor for *E. coli* cells (Ramet et al. 2002). A more general survey of the function of the same number of genes was carried out by Kiger *et al* who assayed for defects in “cell morphology” using automated fluorescence microscopy (Kiger et al. 2003). Lum *et al* were the first to use a transcriptional reporter to measure the output of a signalling pathway, a paradigm that has proved popular. They surveyed the effect of knock-down of 40% of predicted *Drosophila* genes on transcription from a hedgehog pathway reporter, identifying two new components of the pathway (Lum et al. 2003).

The first genome-wide RNAi screen in *Drosophila* was for genes affecting growth and viability (Boutros et al. 2004). The GenomeRNAi database of RNAi screening results in *Drosophila* (<http://rnaï.dkfz.de>) lists 21 genome-wide screens in *Drosophila* cell culture. Many of these screens focus on finding genes involved in various signalling pathways, infection/phagocytosis or the cell cycle: phenotypes it would be harder to study at the

organismal level.

A recent screen with relevance to the work here is a screen of the *Drosophila* genome for genes involved in DNA damage induced apoptosis. This screen identified genes connected with a wide range of cellular processes including basic metabolism. The conservation of the function of several of these genes was also demonstrated in mammalian cells (Yi et al. 2007).

1.2.5.3 Screening in mammalian cell culture.

Screening in mammalian cell culture has mainly focused on screens in human cells. Screens have been conducted on a number of scales and using a range of paradigms. One of the earliest screens was a screen of kinases for genes that modulated the TRAIL-induced apoptosis pathway (see section 1.2.5). This screen employed a library of around 500 siRNAs targeting the kinases along with a few candidate genes, each siRNA targeting a gene, which were transfected in duplicate. One duplicate was then treated with the apoptosis-inducing ligand TRAIL, and the sensitivity of the cells to TRAIL was measured by comparing the viability of treated and untreated cells 24 hours later. Several genes were identified in this way, including several genes previously unassociated with the pathway and one gene of no previous known function. However, only a very small number of genes were tested with more than one siRNA, which makes it difficult to assess the accuracy of the screen (Aza-Blanc et al. 2003).

The first shRNA-mediated screens were published back to back. Paddison *et al* sought to validate their shRNA library and took a clone-by-clone approach, co-transfecting about 7,000 shRNAs with a construct designed to measure proteasome activity and a transfection marker. In this way they identified genes involved in proteasome function, including about 50% of the shRNAs expected to alter proteasome function (Paddison et al. 2004b). In contrast, Berns *et al* used a pooled selection to identify genes that allowed escape from a p53-induced growth arrest. They transduced cells with pools of viruses containing shRNAs targeting 96 genes. A p53-mediated arrest was then induced, escaping colonies were identified, and their shRNA content determined by sequencing. In this way they tested more than 7,000 genes and identified one known and five new modulators of p53-mediated growth arrest. All their hits were confirmed by multiple shRNAs. (Berns et al. 2004).

One common way in which RNAi has been implemented as a screening tool, while keeping costs and labour down, has been to test sets of candidate genes. For example, in one study, 257 growth regulated genes were tested for their ability to differentially affect the

viability of two cell lines (Machida et al. 2006), revealing that 25% of the genes tested affected viability in one but not both cell lines. Using a small set of candidates allows for a more detailed examination of the phenotype studied. Simpson *et al* used 97 siRNAs targeting 37 putative membrane trafficking genes in a high-content microscopy screen for genes involved in secretion (Simpson et al. 2007). Candidate gene sets can be small, such as in a screen for components of the mammalian retromer which screened 30 nexins (Wassmer et al. 2007) or large, such as a screen for modulators of Glutamatergic and GABAergic synapse development using esiRNA pools targeting 160 genes up or down regulated during rodent synapse development (Paradis et al. 2007). When using candidate gene sets the expectation is that the chance of generating hits is increased.

Although candidate gene studies have been successful in confirming the involvement of genes in a variety of processes, the speed of RNAi promises the ability to identify previously unsuspected genes. Many screens focus on a particular subset of the genome, such as the kinases. shRNAs targeting the kinases have been used in a selective screen for genes involved in RAS signalling. In this screen a cell line which is arrested upon Ras induction was transduced with pools of shRNAs, each targeting 96 genes. shRNAs in colonies which escaped arrest upon RAS induction were determined. In this way two known and one novel RAS-regulated kinase was identified (Nicke et al. 2005). Screens of subsets of the genome have also been carried out using a gene-by-gene approach, such as a screen targeting 650 kinases and 222 phosphatases for genes which affect the sensitivity of cells to various apoptosis inducers (MacKeigan, Murphy & Blenis 2005). High-content automated microscopy has also been used with targeted sets of shRNAs. Moffat *et al* screened a set of 1,028 genes including kinases, phosphatases, tumour suppressors and DNA-binding/modifying genes for effects on mitotic progression using a histone modification specific antibody (Moffat et al. 2006)

Pooled selections aid the throughput of larger, unbiased, genome-scale screens. Screens using both a clonal selection and barcoding paradigm have been performed (see section 1.2.5.1, Figure 1.5). A pooled, clonal selection of 8,500 genes revealed a new gene involved in all-trans retinoic acid (ATRA)-induced cell growth arrest. Here, cells were infected with a library of 43,800 shRNAs targeting 8,500 genes and then grown on ATRA containing soft agar media. Colonies which grew despite the presence of ATRA were divided into those that expressed a GFP marker contained on the shRNA vector and those that did not, allowing the removal of colonies that grew owing to spontaneous mutation. Hairpins from the GFP positive colonies were identified. Positive hairpins were re-transfected

individually to confirm their effect. However, no new hairpins were synthesised and so the hits are based on results from only one hairpin per gene (Hattori et al. 2007). Another pooled clonal selection involved examining the ability of pools of shRNA encoding viruses targeting a total of 4,000 genes to transform primary human cells. This screen identified two known and one novel gene, the knock-down of which transformed cells with a similar efficiency to over-expression of oncogenic RAS (Kolfschoten et al. 2005). Here the result was confirmed with multiple shRNAs targeting the same gene. shRNA libraries have also been used to carry out barcode screens. Westbrook *et al* used a barcoding strategy to look for genes, knock-down of which would induce anchorage independent growth in large-T and telomerase-immortalised primary cells. This identified a transcriptional repressor, REST, as a tumour repressor. Results were confirmed using multiple hairpins, and a sequencing of colonies based approach which closely matched the barcode array results, validating the use of barcode arrays, where previously only proof-of-principle experiments had been published (Westbrook et al. 2005).

Although pooled strategies are very attractive when dealing with such large sets of reagents, they do limit the phenotypes that can be studied. The use of one well one gene approaches increases the phenotypes that can be studied. One screen introduced shRNAs along with a GFP marker into cells and used time-lapse microscopy used to track the motility of transfected cells. In this way CUTL1 was identified as a target of TGF β signalling and as being involved in invasion and metastasis (Michl et al. 2005).

siRNA libraries have been employed in large genome-scale screens in mammalian cells. Examples include screens for genes that are required for tumour-cell survival (Morgan-Lappe et al. 2007) and genes that prevent differentiation of mesenchymal stem cells into osteoclasts (Zhao, Ding 2007). siRNAs libraries have also been employed for essentially genome-wide screens targeting nearly all genes in the human genome. One such screen tested the effect of gene knock-down on sensitivity of cells to the cancer drug paclitaxel (Whitehurst et al. 2007). Such designs allow the gathering of phenotypic information on a quantitative rather than qualitative basis.

However, this can present a problem for “hit” selection, particularly since such large quantities of data are involved. In this case a very stringent statistical hit selection was applied. The screen was carried out in sextuplicate, with three replicates treated and three untreated. This allowed the application of a student’s t-test to test the significance of the difference between the treated and untreated replicates. After the application of a correction for multiple testing, genes that gave a significant difference and also were in the top 2.5

centile-rank for absolute size of the treated/untreated ratio were selected as hits. This still left 87 hits, only 6 of which were selected for confirmation and follow up. Another genome-wide screen employed high-content microscopy to examine the effects of gene knockdown on cell-cycle progression. Information on a number of phenotypic parameters was recorded and the screen was carried out in duplicate. A total of 1,152 genes were reported to reproducibly alter at least one of the phenotypes studied. Although 24 of these genes were carefully confirmed with the use of multiple siRNAs, the authors mainly tried to avoid selecting genes for follow up by employing systems-level analysis. They found that their hits clustered into 8 phenotypic groups, and that they could combine the information from the screen with information from the literature or from published interaction experiments to build phase specific networks. (Mukherji et al. 2006).

Several groups have published protocols and proof-of-principle experiments for cell-array based RNAi screens, but no novel screens have yet been published using this technique (Bailey et al. 2006, Erfle et al. 2007, Silva et al. 2004).

Selecting genes for follow up can be approached in several ways. In many of the pooled shRNA screens, the number of hits seems to be small enough that all novel hits can be confirmed. For screens which produce a larger number of hits, genes are often selected on the basis of biological interest (e.g. Aza-Blanc et al. 2003, Whitehurst et al. 2007, Zhao, Ding 2007). Although this may yield biologically interesting results, it seems a shame given the ability of RNAi screening to identify genes in an unbiased manner.

Given the controversy over the specificity of RNAi (see section 1.2.3), it is perhaps surprising that many screens are published in which hits are not confirmed by multiple siRNAs targeted against the same gene (Hattori et al. 2007, Zhao, Ding 2007). At best, large screens tend only to verify a few genes that they select for follow up (Mukherji et al. 2006, Westbrook et al. 2005, Whitehurst et al. 2007). The danger of not confirming hits with multiple siRNAs is shown by the rigorous confirmation process undertaken by Morgan-Lappe *et al* in their screen for genes which reduce the viability of tumors cells. Of 48 genes selected as hits only 23 were confirmed on retesting. Of these, only 10 confirmed with multiple siRNAs in multiple assays and of these only 3 had phenotypes that correlated with the level of knock-down (Morgan-Lappe et al. 2007). Others have found that their screens have returned only hits that are due to off-target effects (Lin et al. 2005, Lin et al. 2007).

RNAi screening has yielded new insights in many areas. There are multiple ways that screening can be carried out, depending on the system under study, using a range of reagents. Whichever method is chosen, careful strategies are necessary to confirm hits and to select

genes for follow-up studies.

1.3 TRAIL-induced apoptosis

Tumour Necrosis Factor (TNF)-Related Apoptosis-inducing Ligand (TRAIL) was originally identified using searches of EST databases for homology to other members of the TNF family by two groups independently and shown to have apoptosis-inducing activity (Pitti et al. 1996, Wiley et al. 1995). The TRAIL ligand is a type II membrane-bound protein, although it can also be expressed as a soluble ligand, and is trimeric. It is thought to have a number of roles in the immune system (see 1.3.4). The ligand is widely expressed in almost all tissues (Wiley et al. 1995), as are its receptors (Pan et al. 1997b, Walczak et al. 1997). This suggested that the regulation of its apoptosis-inducing activity must be different to that for the FAS system, where expression of both ligand and receptor is tightly controlled. TRAIL is interesting as it induces apoptosis in between a half and two-thirds of tumour cells but not normal cells (Ehrhardt et al. 2003, Walczak et al. 1999, Zhang et al. 1999).

The mechanism by which the sensitivity of cells to TRAIL is regulated is of interest for two reasons. Firstly, by understanding the mechanism of sensitivity, we can predict which cell types – tumour and normal – will be sensitive. This will aid decisions as to the effectiveness of TRAIL as an anti-cancer agent. It will also help to identify combination treatments that increase the effectiveness of TRAIL. Secondly, understanding the regulation of TRAIL sensitivity gives an insight into why so many tumour cells are sensitive when the molecular basis of their transformation is so diverse.

Below is reviewed the current state of knowledge on the mechanism of TRAIL-induced apoptosis and the regulation of sensitivity to TRAIL. A brief overview of the physiological function of TRAIL and the prospects for its use as an anti-cancer treatment is also given.

1.3.1 Mechanism of TRAIL-induced apoptosis

The mechanism of apoptosis induction by the TRAIL ligand is similar to that of other TNF family apoptosis-inducing ligands, particularly that of the FASL/Apo1L/CD95L ligand (Reviewed: Ashkenazi, Dixit 1999) with ligand-bound receptor triggering apoptosis through both a caspase cascade and via the mitochondrial associated intrinsic pathway. The pathway is summarised in Figure 1.6.

The TRAIL ligand can bind four membrane-bound TNF family receptors – DR4 (also known as Death Receptor 4, TRAIL-R1, and TNFRSF10A), DR5 (Death Receptor 5,

TRAIL-R2 or TNFRSF10B), DcR1 (Decoy Receptor 2, TRAIL-R3, TNFRSF10C, TRID or LIT) and DcR2 (Decoy Receptor 2, TRAIL-R4, TNFRSF10D, TRUNDD) (Degli-Esposti et al. 1997a, Degli-Esposti et al. 1997b, Pan et al. 1997a, Pan et al. 1997b, Walczak et al. 1997). TRAIL can also bind the soluble protein Osteoprotegerin (OPG) (Emery et al. 1998). Of these 5 receptors, only DR4 and DR5 contain complete death domains, and it is the binding of the TRAIL ligand to these receptors that induces apoptosis (Pan et al. 1997b, Walczak et al. 1997).

The binding of TRAIL to either DR4 or DR5 causes recruitment of the FADD adaptor protein via interaction of the death domains of the receptors and those of the FADD protein. Recruitment of FADD in turn leads to the recruitment of the cysteine-protease Caspase-8 (also known as FLICE or MACH)(Kuang et al. 2000) through interactions between the Death Effector Domains (DED) of FADD and Caspase-8. Recruitment of Caspase-8 results in the processing from the long inactive form, termed procaspase-8, to the active form, which involves two cleavage events, separating the long and short active domains from one another and from the pro-domain. The complex of Death receptor, FADD and Caspase-8 is termed the DISC (Death Inducing Signalling Complex).

The activated DISC can induce apoptosis by both the extrinsic and intrinsic pathways (Suliman et al. 2001). The extrinsic pathway involves the cleavage and activation of the executioner caspases such as Caspase-3 and Caspase-7. The inactive form of procaspase-3 is cleaved by caspase-8 to give p24 and p12 caspase-3 subunits. The p24 subunit is further self-processed to give the p19/p17 active subunit. (Martin et al. 1996).

The intrinsic pathway is activated by the cleavage of Bid by Caspase-8, which leads to mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c and DIABLO (also known as Smac), a process for which the protein BAX is required (Deng, Lin & Wu 2002, Suliman et al. 2001). Cytochrome c associates with caspase-9 and APAF-1 to form the apoptosome. The apoptosome activates caspase-9, which in turn activates procaspase-3. However, Caspase-9 activation has been found to be dispensable for TRAIL-induced apoptosis (Deng, Lin & Wu 2002), and therefore is not considered part of the TRAIL-induced apoptosis mechanism. DIABLO functions to promote the self-processing of p24 Caspase-3 to the active p20/p17. This is achieved by the binding of DIABLO to XIAP, a member of the IAP (inhibitor of apoptosis protein) family, which would otherwise bind Caspase-3 and inhibit its self-activating activity (Deng, Lin & Wu 2002). Thus blockage of the intrinsic pathway can delay or prevent TRAIL-induced apoptosis, possibly depending on the level of XIAP (Deng, Lin & Wu 2002, Suliman et al. 2001).

1.3.2 Regulation of TRAIL sensitivity

Since it was demonstrated that most transformed, but not normal, cells are sensitive to TRAIL-induced apoptosis, much research has addressed the question of how sensitivity to TRAIL is regulated. This intensified when it was found that up to 50% of tumour cells are resistant to TRAIL (Ehrhardt et al. 2003, Zhang et al. 1999). The TRAIL ligand is widely expressed in many normal tissues, suggesting that sensitivity is expressed at a level other than ligand expression. Interest has focused on five areas: Regulation of the TRAIL receptors, the so-called decoy receptors, the inhibitory molecule c-FLIP, the expression of IAP proteins, and the effects of other signalling pathways.

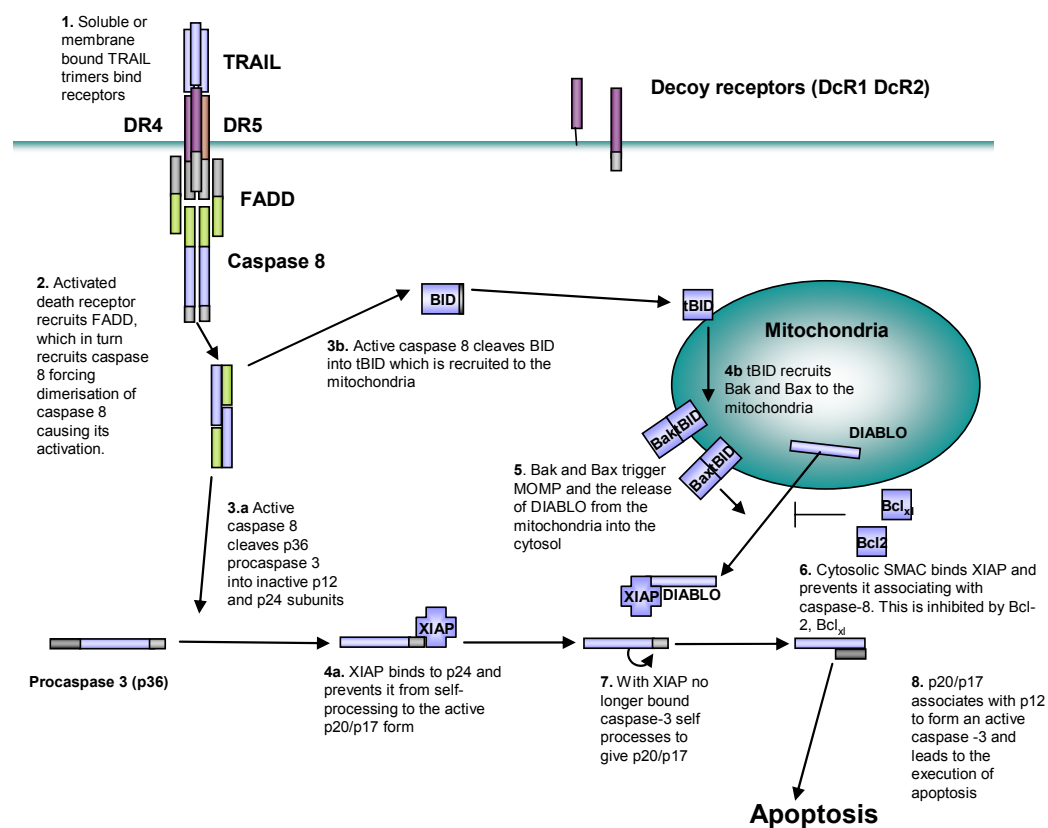


Figure 1.6 A simplified schematic of events leading to TRAIL-induced Apoptosis

1.3.2.1 Regulation of the TRAIL receptors

The original TRAIL receptor cloning papers found that both receptors were widely expressed in a range of normal tissue at the RNA level (Pan et al. 1997b, Walczak et al. 1997). However, studies have looked at the relationship between the levels of the two receptors and the sensitivity of cells to TRAIL. Kim *et al* found that levels of DR4, but not

DR5, correlated with the level of apoptosis induced by TRAIL in a panel of sensitive and insensitive tumour cell lines, but that this could not completely explain sensitivity in all cases (Kim et al. 2000). The same study also found that resistance to TRAIL in one case could be explained by a mutation in the DR4 gene. Resistance in tumour cells can also be explained by deletion of the DR4 gene (Zhang et al. 1999).

This stands in opposition to the finding that in lung carcinoma cells a mutant TRAIL selective for DR4 was less able to induce apoptosis than a DR5-specific TRAIL ligand mutant. This finding was replicated in several tumor cell lines leading the authors to conclude that DR5 was more important in the induction of apoptosis than DR4 (Kelley et al. 2005). However, this study had several problems. Firstly, the DR4-specific ligand had reduced affinity for DR5, but not for the decoy receptors (see below), but the DR5 specific ligand had reduced affinity for all the TRAIL receptors except DR5. Secondly, all the cell lines used in the study expressed DR5 at a higher level than DR4. Indeed, it has been demonstrated, using receptor-sensitive TRAIL mutants, that some sensitive cells signal purely through DR4 (MacFarlane et al. 2005) and so it is likely that the relative importance of the two receptors is cell-type specific.

Expression of DR5 can be regulated by p53 (Takimoto, El-Deiry 2000) and indeed resistant tumor cells can be sensitised to TRAIL by cellular stressors such as γ -irradiation, or anti-cancer drugs such as etoposide, 5-Fluorouracil and proteasome inhibitors in both p53-dependent and p53-independent manners (Anan et al. 2006, Chinnaiyan et al. 2000, Frese et al. 2003, Ganten et al. 2004, Ganten et al. 2005, Gibson et al. 2000). Sensitisation by these agents results in increased expression of either DR5 alone or both DR5 and DR4. However, one report has demonstrated that this increase in receptor expression is dispensable for the sensitisation (Ganten et al. 2004). These treatments do not sensitise normal cells, which generally already express TRAIL receptors, both at an mRNA and a protein level (Ganten et al. 2005, Pan et al. 1997b, Walczak et al. 1997, Wu, Ogawa & Kakehi 2004).

One explanation for these discrepancies could be the localisation of the receptors. Stimulation of natural killer cells (NK cells) and CD8⁺ T cells leads to movement of TRAIL receptor molecules from the cytoplasm of resting NK and CD8⁺ T cells to the surface of stimulated cells. However, these cells are still TRAIL resistant (Mirandola et al. 2004). Zhang *et al* have shown that although a panel of melanoma cells lines, expressing all the TRAIL-receptors at both the mRNA and protein level, have a wide variability in the sensitivity to TRAIL, there was a correlation between the level of surface expression of the receptors and the degree of apoptosis induction in sensitive cell lines. Interestingly, the localisation of DR4

and DR5 is controlled in different ways, with expression of DR4, but not DR5, on the cell surface being dependent on the signal recognition particle (SRP) (Ren et al. 2004).

It would therefore seem that, since both sensitive and insensitive cells express the TRAIL receptors on their surface, this does not provide a conclusive explanation of why transformed cells, but not normal, cells are sensitive to TRAIL. However, modulation of TRAIL receptor surface expression does appear to provide a mechanism for the resistance of some tumour cell lines.

1.3.2.2 The TRAIL decoy receptors

Of the four receptors that the TRAIL ligand binds only two are capable of transmitting an apoptosis-inducing signal. This has led to the remaining receptors being termed “decoy receptors” based on the hypothesis that their function is to titrate away TRAIL from the death-inducing receptors. DcR1 and DcR2 both share homology to the two apoptosis-inducing receptors in their extra-cellular domains. However, DcR1 completely lacks an intracellular domain and is anchored to the cell membrane via a phosphatidylinositol glycine (GPI) anchor (Degli-Esposti et al. 1997b, Pan et al. 1997a), and DcR2 has a truncated, inactive death-domain (Degli-Esposti et al. 1997a). The final “decoy” receptor is OPG, which is soluble and contains no death domain (Emery et al. 1998).

Overexpression of any one of the non-apoptosis-inducing ligands can protect sensitive cells from the cytotoxic effects of TRAIL (Degli-Esposti et al. 1997a, Degli-Esposti et al. 1997b, Emery et al. 1998, Pan et al. 1997a). Although this was originally assumed to be due to a titration of the TRAIL ligand away from the death-inducing receptors, other mechanisms have been proposed. Mérimo *et al* have suggested that DcR1 and DcR2 function in different ways. They have demonstrated that due to its GPI attachment to the membrane, DcR1 is mainly found within lipid rafts and that in cells over-expressing this receptor TRAIL ligand is also mainly found in lipid rafts, while DR4 and DR5 are excluded. When they over-expressed DcR2 however, they found that it was recruited to the DISC upon TRAIL treatment and that DR4 was excluded. They found that Caspase-8 was recruited to this DR5/DcR2 DISC, but not activated (Merino et al. 2006). Some groups have found that binding of the TRAIL ligand to the DcR2 receptor can trigger the NF- κ B pathway, which could lead to an increase in the expression of anti-apoptotic factors (Degli-Esposti et al. 1997a). This could suggest a wider role for DcR2 in TRAIL-mediated pathways.

However, these and other studies into the functions of the non-death-inducing ligands have relied upon overexpression, and so may not necessarily reflect the physiological

role of these receptors. Although some reports have claimed that normal cells express higher levels of the decoy receptors than transformed cells (Pan et al. 1997a), others have not found a correlation between sensitivity and decoy receptor expression either at the mRNA level (Kim et al. 2000) or expression on the cell surface (Zhang et al. 1999). Furthermore, several of the drug treatments that sensitise resistant tumour cells to TRAIL also increase expression on the surface of the decoy receptors (Ganten et al. 2004, Ganten et al. 2005).

Since studies showing the protective effect of decoy receptors rely on over-expression systems and no correlation has been found between sensitivity and expression of these receptors, the regulatory role of decoy receptors in a physiological situation remains unclear.

1.3.2.3 Bcl -2 and Bcl-XL

Bcl-2 and Bcl-XL are general anti-apoptotic factors which inhibit the action of Bax in inducing MOMP (Figure 1.1). Several overexpression studies have shown little effect of Bcl-2 and Bcl-XL on TRAIL-induced apoptosis (Gazitt, Shaughnessy & Montgomery 1999, Walczak et al. 2000), nor does BCL-2 levels correlate with TRAIL sensitivity, although they were isolated in an expression screen for genes involved in TRAIL-induced apoptosis (Burns, El-Deiry 2001).

1.3.2.4 The cFLIP protein

Cellular FLICE Inhibitory Protein (cFLIP, also known as CFLAR, Usurpin, Casper or FLAME) was first cloned using its homology to Caspase-8 (Rasper et al. 1998). cFLIP is expressed as at least three different isoforms: cFLIP_S, cFLIP_R and cFLIP_L (Golks et al. 2005, Irmeler et al. 1997, Rasper et al. 1998). The cFLIP_S and cFLIP_R proteins contain two DED domains, homologous to those in both Caspase-8 and FADD, and short N-terminal domains. In addition to the DED domains, cFLIP_L contains a catalytically inactive caspase-like domain (Golks et al. 2005, Irmeler et al. 1997).

Overexpression of cFLIP protects cells from the apoptosis-inducing ligand FAS by associating with Caspase-8 and FADD and preventing the assembly/activity of the DISC (Golks et al. 2005, Irmeler et al. 1997, Rasper et al. 1998). Furthermore, siRNA mediated knock-down of cFLIP sensitises otherwise resistant NK cells to TRAIL-induced apoptosis (Mirandola et al. 2004) and mouse embryonic fibroblasts from cFLIP^{-/-} mice have an increased sensitivity to FAS. However, there have been some reports that cFLIP_L might actually activate Caspase-8 in some situations (Micheau et al. 2002).

Expression of cFLIP is widespread in most normal tissues, although expression was not detected in colon, placenta and testis samples (Rasper et al. 1998). High levels of cFLIP have been found to correlate with resistance to TRAIL-induced apoptosis in tumour cells. One study that found that 5 out of 6 TRAIL-resistant transformed cell lines expressed cFLIP mRNA, but only 1 out of 5 sensitive lines expressed FLIP mRNA (Kim et al. 2000). However, a separate study found that few melanoma cells lines expressed cFLIP protein, irrespective of their TRAIL sensitivity, and that there was no correlation between protein expression levels and sensitivity (Zhang et al. 1999).

Sensitisation of cells to TRAIL via treatment with 5-FU involves a reduction both in cFLIP protein levels and its recruitment to the DISC (Ganten et al. 2004). In contrast, siRNA mediated knock-down of cFLIP leads to further sensitisation of cells treated with proteasome inhibitors, arguing that these compounds are acting through a different mechanism (Ganten et al. 2005).

Given that cFLIP has been shown to inhibit apoptosis and that its knock-down can sensitise cells to TRAIL-induced apoptosis, it would appear that cFLIP is an important regulator of sensitivity to apoptosis. Although not all studies have shown a correlation between cFLIP levels and sensitivity to TRAIL, this demonstrates that this is not the only mechanism by which cells can become resistant to TRAIL, not that it is a physiologically unimportant one. However, cFLIP is a general inhibitor of apoptosis and its effects are not restricted to TRAIL-induced apoptosis and so cannot entirely explain the sensitivity of some cells, but not others, to TRAIL-induced apoptosis when other ligands, such as FAS do not follow this pattern.

1.3.2.5 Inhibitor of Apoptosis Proteins (IAPs)

IAPs are characterised by the presence of BIR domains (baculoviral IAP repeat) and RING zinc-finger domain. XIAP, cIAP1 and cIAP2 bind directly to the executioner caspases and inhibit the processing of the pro-caspase form to the active form (Deveraux et al. 1997, Roy et al. 1997). Over-expression of these can lead to a general resistance to apoptosis (Deveraux et al. 1997). The RING zinc-finger domain is also involved in the ligation of ubiquitin to caspases therefore leading to their degradation (Suzuki, Nakabayashi & Takahashi 2001).

The IAP Survivin has been associated with resistance to TRAIL in renal cell carcinoma (Griffith et al. 2002) and overexpression of Survivin in sensitive, Survivin-negative cells can lead to resistance. Survivin is predominantly expressed during the G2/M phase of

the cell cycle and has been demonstrated to have a role in the control of the cell cycle. It co-localises with several microtubule associated factors, and knock-out mice show a failure of cytokinesis (Li et al. 1998, Uren et al. 2000). It has been suggested that Survivin may form a link between cell-cycle control and cell death with its expression during G2/M preventing a default cell death activation (Li et al. 1998). The role of BIR domain containing proteins in cell cycle regulation is conserved in a group of BIR containing proteins in the worm and in yeast. Worm embryos deficient in the protein BIR-1 are unable to compete cytokinesis and the null phenotype is indistinguishable from that of Aurora kinase null embryos (Fraser et al. 1999, Speliotes et al. 2000).

1.3.2.6 Regulation of TRAIL sensitivity by other signalling pathways

It is known that several other signalling pathways are connected to the TRAIL pathway. The NF- κ B, RAS/MAPK/ERK, MYC, PKC and ATK/PKB pathways have all been implicated in the regulation of TRAIL sensitivity. Several of these pathways have also been shown to be regulated by the TRAIL pathway, suggesting a complex, interconnected regulatory network whose output is the decision to live or die.

The ligation of the TRAIL ligand to any of its receptors, with the exception of DcR1 and OPG, can lead to the activation of the NF- κ B transcription factor in a process that involves the recruitment of the death domain kinase RIP to the DISC (Degli-Esposti et al. 1997a, Lin et al. 1999). Treatment of cells with proteasome inhibitors leads to a reduction in the activity levels of NF- κ B. However, whether or not this is the cause of proteasome inhibitor-mediated sensitisation to TRAIL is controversial (Ganten et al. 2005, Ravi et al. 2001). NF- κ B activity can also sensitise cells to TRAIL. This is accompanied by an up regulation of TRAIL receptor expression. The balance of pro- and anti-apoptotic signals is thought to be regulated by the ratio of c-Rel and RelA in the NF- κ B dimer (Ravi et al. 2001)

Most studies into the sensitivity of cells to TRAIL have been conducted using either sensitive or resistant transformed cells lines, and as such we know more about the resistance of certain transformed cells to TRAIL than we do about the sensitivity of transformed cells compared with normal cells. Several groups have approached this problem using a system that involves creation of transformed cell lines from normal cells using defined genetic changes (Nesterov et al. 2004, Wang et al. 2004/5).

Immortalisation of HEK or fibroblast cells with the early region of the SV40 virus and a constitutively active telomerase does not sensitise these cells to TRAIL. However, the addition of an oncogenic mutant RAS transforms to these immortalised cells and sensitises

them to TRAIL-induced apoptosis. The surface expression of DR5, and thus the recruitment of Caspase-8 to the DISC, is also increased, suggesting a possible mechanism (Nesterov et al. 2004). A constitutively active MEK could substitute for oncogenic RAS in the sensitization, showing that the MAPK/ERK pathway was important down-stream of RAS. ERK2 signalling is required for the PG490-mediated sensitisation of resistant tumour cells (but not normal cells) to TRAIL (Frese et al. 2003). At the same time it was shown that over-expression of MYC could also sensitise immortalised fibroblast cells to TRAIL-induced apoptosis *in vitro* and *in vivo*. This also leads to the up regulation of DR5 expression on the cell surface (Wang et al. 2004/5). It has also been shown that stabilisation of MYC by knock-down of GSK3 β or FBW7 leads to sensitisation (Rottmann et al. 2005).

It has been suggested that RAS sensitises cells to TRAIL by stabilising the MYC protein. This is supported by the fact that siRNA mediated knock-down of MYC removes the sensitizing effect of oncogenic RAS (Wang et al. 2005). Against the model that sensitisation is induced by RAS-mediated stabilisation of MYC and therefore an increase in DR5 surface expression is the fact that ERK2-mediated sensitisation of a cell to TRAIL does not involve an increase in DR5 surface expression (Frese et al. 2003). Indeed, as discussed above, some normal, insensitive, cell types have been shown to express DR5 on the surface. However, both RAS and MYC function through other mechanisms. Overexpression of both MYC and oncogenic RAS leads to a slight increase in levels of Caspase-8, FADD and BID in addition to DR5 (Nesterov et al. 2004, Wang et al. 2004/5). MYC also directly represses the transcription of cFLIP (Ricci et al. 2004), while RAS activation inhibits the translation of cFLIP via a RalA/cdc25 pathway (Panner et al. 2006).

Although a correlation has been shown between MYC levels and TRAIL sensitivity in TRAIL-sensitive cell lines, not all sensitive cells showed a high expression of MYC, and not all lines expressing a high level of MYC are sensitive (Ricci et al. 2004). Indeed, not all transformed cells have increased levels of MYC. This can be partly explained by the fact that the effects of MYC on DR5 levels are indirect (Wang et al. 2004/5). This means that it is possible that other effectors can work downstream of MYC.

Other signalling molecules that have been shown to affect the sensitivity of cells to TRAIL include Protein Kinase C (PKC) and the Akt/PKB pathway. PKC has been shown to regulate FADD recruitment to the DISC independently of receptor surface expression or FADD phosphorylation (Harper et al. 2003). Akt levels in transformed cells correlate with TRAIL sensitivity and cells expressing high levels of Akt do not cleave BID upon TRAIL treatment. This finding has been experimentally replicated by using dominant negative or

constitutively active Akt to regulate TRAIL sensitivity. (Chen et al. 2001, Thakkar et al. 2001)

Sensitivity of cells to TRAIL can be regulated at the level of receptor surface expression, decoy receptors and the expression of inhibitory proteins, such as cFLIP. However, perhaps with the exception of cFLIP, these proteins do not seem to be able to mark the difference between resistant, normal cells and sensitive transformed cells. The fact that the resistance of some transformed cells can be explained by modulation of these factors suggests that the resistance of this subset has a different mechanism to the general resistance of normal cells. That is, transformation sensitises cells, some of which can escape by a secondary, acquired mechanism, such as losing expression of the TRAIL receptor. This is supported by the observation that many treatments which sensitise resistant transformed cells to TRAIL do not sensitise normal cells to TRAIL. Although cFLIP is widely expressed in normal cells, and high levels in tumour cells correlates with resistance, cFLIP inhibits apoptosis induced by any Caspase-8 dependent stimulus, and cannot explain, for instance, the sensitivity of some cells to FAS that are resistant to TRAIL. Many pathways involved in transformation have been implicated in TRAIL sensitivity, including NF- κ B, RAS and MYC. However, the effects of these appear to be cell-type specific, and function through regulation of the same factors already discussed, although other, unknown mechanisms may exist. As such, they cannot provide an explanation as to why transformed cells generally are sensitive as changes in these pathways are not common to all transformed cell types. This leaves two options: 1) All pathways that lead to transformation regulate TRAIL sensitivity via independent mechanisms or 2) Some factor, common to all transformed cell types, remains to be found. This makes the regulation of TRAIL sensitivity an interesting and important problem to study.

1.3.3 Genes involved in TRAIL-induced apoptosis

The collation of information from the literature on the mechanism of TRAIL-induced apoptosis, work on determinants of the sensitivity of cell to TRAIL-induced apoptosis, and genes identified in the Aza-Blanc screen allows the production of a list of genes which, when knocked-down, should block TRAIL-induced apoptosis in sensitive cells. This list is presented in Table 1-2. The genes, which have been selected from the Aza-blanc screen, but are not reported elsewhere in the literature, are shown in a separate column since the lack of rigorous confirmation of hits in this study means the results must be treated with caution.

<i>Confirmed positive controls</i>	<i>Controls from (Aza-Blanc et al. 2003)</i>
TNFRSF10A (DR4)	BLK
TNFRSF10B (DR5)	PKM2 like
FADD	GSK3A
Caspase-8	CCDC139 (FLJ32312)
Caspase 3	FBXO11 (FLJ12673)
Bax	ROS1
Bid	ABL2
DIABLO (SMAC)	PRKRIR (DAP4)
SRP72	MAPK10 (JNK3)
MYC	TCF4
GSK3 β	VPS16
	GUK1
	PRKCQ
	PRKAA2
	WDFY4 (FLJ00156)
	PRKCD
	IRAK1
	DVL2

Table 1-2 Genes involved required for TRAIL induced apoptosis

Table shows genes reported to be involved in TRAIL mediated apoptosis, which when knocked-down could be expected to protect cells from TRAIL-induced apoptosis. Genes from the general literature are separated from genes reported in Aza-Blanc *et al*, owing to the lack of confirmation of genes from the screen. Names presented are HUGO names for genes, names in brackets are the names originally reported by Aza-blanc *et al*, or other commonly used names for the gene.

1.3.4 The physiological role of TRAIL

Although the cytotoxic effect of TRAIL against transformed cell lines has been intensively studied, less is known about the physiological role of TRAIL. Homozygous TRAIL knockout (TRAIL^{-/-}) mice do not show any grossly abnormal phenotype. They have normal tissue architecture, lymphoid cell homeostasis and bone density (Sedger et al. 2002). TRAIL does appear to function in both the innate and adaptive immune system, and has some role in tumour surveillance and anti-viral responses. There is also possibly conflicting data regarding TRAIL's involvement in auto-immune reactions and the maintenance of immune privileged sites.

Many cell types in the innate immune system up-regulate the expression of TRAIL upon stimulation, including monocytes, dendritic cells (DCs) and natural killer (NK) cells (Ehrlich et al. 2003, Sato et al. 2001, Takeda et al. 2001). There is also evidence that these cells exert their cytotoxic effect, especially against tumour cells, via TRAIL (Kayagaki et al. 1999, Kemp, Elzey & Griffith 2003, Liu et al. 2001, Sato et al. 2001, Takeda et al. 2001). This is particularly important for NK cells as this is their main function in the body. Indeed, liver NK cells express TRAIL constitutively (Takeda et al. 2001).

Although T and B cells can also express TRAIL upon stimulation and cytotoxic T

cells have been shown to exert their killing effect through TRAIL (Ehrlich et al. 2003, Janssen et al. 2005, Kayagaki et al. 1999), TRAIL also seems to have a role in the regulation of cells from the adaptive immune system. Helpless CD8⁺ T cells, which do not require priming by CD4⁺ T cells, do not undergo an expansion upon encountering their stimulating antigen a second time. This secondary expansion is suppressed by TRAIL and blocking TRAIL allows a second expansion to take place (Janssen et al. 2005).

The involvement of TRAIL in the cytotoxicity of immune cells against tumour cells indicates a role for TRAIL tumour surveillance mechanisms. TRAIL^{-/-} mice do not have an increased incidence of tumours at an early age (Sedger et al. 2002), but they do have an increased risk of lymphomas if aged for a much longer period (Zerafa et al. 2005). Further, a lack of TRAIL in p53^{+/-} mice leads to an increase in lymphomas and carcinomas. TRAIL^{-/-} mice also show increased growth and metastasis of introduced tumours (Cretney et al. 2002, Sedger et al. 2002, Takeda et al. 2001). In one case it was demonstrated that this effect was due to the NK cells rather than cells of the adaptive immune system (Sedger et al. 2002). Consistent with this, the growth and metastasis of mammary tumours in a Her2/neu background was unaffected despite sensitivity to TRAIL. These tumours were MHC class I expressing and therefore would not have been subject to NK cell killing.

The issue of TRAIL's involvement with tumorigenesis is somewhat complicated by the finding that in certain situations TRAIL can promote tumour growth and metastasis. One study found that 50% of freshly isolated leukemia cell lines were resistant to TRAIL and that in a subset of these TRAIL actually reduced apoptosis and even promoted proliferation (Ehrhardt et al. 2003). *In vivo* work has also shown that TRAIL-resistant xenograft tumours in SCID mice are induced to metastasise by TRAIL treatment (Trauzold et al. 2006). Both groups found that this was mediated through TRAIL's ability to activate the NF- κ B pathway, a pathway that can itself be pro- or anti-apoptotic (see 1.3.2.6). Thus it seems that TRAIL can induce a "proliferate or die" signal depending on the apoptotic sensitivity of the cell.

As well as its role in tumour surveillance, TRAIL also has an anti-viral role. Depleting TRAIL in mice that were infected with EMCV increased viral load (Sato et al. 2001) and is equivalent to the effect of depleting NK cells. It has also been shown that HIV-infected T-cells and macrophages are sensitive to TRAIL-induced apoptosis and that treatment with TRAIL reduces production of HIV mRNA and protein (Lum et al. 2001). Again, however, TRAIL seems to have effects in both directions, as TRAIL^{-/-} mice have an enhanced resistance to MCMV infection (Diehl et al. 2004). This was shown to be due to an increased level of IL-2 and interferon- γ , suggesting a role for TRAIL in negative regulation of immune

cells.

Speculation on a possible role for TRAIL in the regulation of cells of the adaptive immune system led to a study of the possible effects of TRAIL on auto-immune disorders. Similar to tumorigenesis, it was found that the rate of spontaneous auto-immune disease was similar in TRAIL^{-/-} mice to wild-type mice, but deficient mice were more sensitive to induced auto-immune disorders, including collagen-induced arthritis and streptozotocin-induced diabetes (Lamhamedi-Cherradi et al. 2003). Systematic administration of TRAIL can reduce the effects of experimental autoimmune encephalomyelitis (EAE), an experimentally induced model of multiple sclerosis in which immune cells infiltrate the central nervous system (CNS) leading to the destruction of the myelin around axons (Hilliard et al. 2001). Interestingly, direct blockage of TRAIL in the brain by injection of a modified TRAIL blocking, soluble TRAIL receptor leads to a reduction in the severity of the disease (Aktas et al. 2005), suggesting a dual role for TRAIL: firstly in regulating auto-immune T-cells and secondly in action of auto-immune T-cells on their targets.

The regulation of auto-immune T-cells in the brain suggests a role for TRAIL in the maintenance of so called immune privileged sites. The brain has very low numbers of T-cells and is one of a number of sites where cells of the immune system are excluded, presumably because the effects of inflammation would be very damaging. The brain does contain a small number of CNS-derived dendritic cells (CNS-DCs). However, unlike normal DCs, CNS-DC do not act to stimulate naive T-cells. Further, they actively block the proliferation of activated T-cells, a function that is dependent on TRAIL (Suter et al. 2003). TRAIL has also been implicated in the establishment of immune privilege at the interface between mother and placenta (Phillips et al. 1999).

This evidence suggests that TRAIL has a multitude of roles, both in the regulation and action of cells of the immune system. In addition to its role in tumour surveillance, it also has roles in anti-viral responses, auto-immune reactions and the establishment of immune privilege. However, the role of TRAIL is often complicated and contradictory. In some cases this is due to its ability to stimulate proliferation via the NF- κ B pathway, in others, to the fact that it is both an effector and a regulator of the immune system.

1.3.5 Clinical prospects for TRAIL

There were initially very high hopes for TRAIL as an anti-cancer drug. Its ability to induce apoptosis in tumour cells, but not normal cells, gave it an obvious advantage over other apoptosis-inducing ligands such as FAS and TNF α , which lead severe effects on

normal tissue. Anti-Fas antibodies cause massive apoptosis of mouse liver cells *in vivo* (Ogasawara et al. 1993). Systemic TNF α , administration leads to a sepsis-like syndrome (Lejeune et al. 2006) and this has limited its use to limb salvage by regional limb perfusion in soft-tissue sarcoma treatment.

Pre-clinical studies showed promising remission of xenograph tumours in nude mice without toxicity (Ashkenazi et al. 1999, Sedger et al. 2002). However, this was challenged by the finding that normal human hepatocytes, not hepatocytes from mice or non-human primates were sensitive to recombinant TRAIL (Jo et al. 2000). It was noted that this study used a his-tagged version of the recombinant TRAIL and also that the native TRAIL dimer contained a zinc ion, while the his-TRAIL had not been optimised for physiological zinc content. Two groups later showed that the toxicity observed could be due to the tag, and that un-tagged recombinant TRAIL with the correct zinc content did not induce this apoptosis in normal cells. (Lawrence et al. 2001, Qin et al. 2001). Another group has confirmed this finding and also shown that hepatocytes are not sensitised to TRAIL on proteasome inhibition (Ganten et al. 2005).

Several TRAIL-based treatments are now in clinical trials (Reviewed in Duiker et al. 2006). Phase I clinical trials with a recombinant TRAIL have been completed and Phase II trials have recently been initiated. As well as TRAIL itself, several trials have been initiated using antibodies which target the TRAIL receptors. HGS-ETR1 targets DR4 and there are positive preliminary results for two phase I and three phase II trials using HGS-ET1 as a treatment for non-Hodgkin's lymphoma, colorectal cancer and non-small-cell lung cancer. HGS-ETR2 targets DR5. Preliminary results from two phase I trials show that although high doses of the antibody can lead to adverse effects including renal failure, possibly due to liver malfunction, lower doses show minimal toxicity. Phase I trials have also been initiated with a second DR5 targeting antibody known as HGS-TR2J.

There is hope TRAIL's ability to synergise with other treatments, such as those that activate the p53 pathway or proteasome inhibitors, may lead to more effective treatments for tumours refractory to either of these treatments alone. So far, the only trials initiated are phase I trials for HGS-ETR1 combined with gemcitabine and cisplatin, and with paclitaxel and carboplatin. Initial results are apparently promising.

1.4 Aims

High-throughput genetic screens represent an important route to add functional annotation to genes identified within the genome. In mammalian cell culture, gene levels can

be increased by expressing open-reading frames from constitutive promoters on introduced plasmid vectors. RNAi presents a novel way to reduce gene function on a large scale. These techniques present us with the tools necessary to carry out genome-scale screens in mammalian cell culture.

The TRAIL apoptosis system is an interesting and medically important pathway which is amenable to study using such genome-scale gene-perturbation studies. Previous experiments have shown that the pathway can be studied using over-expression and RNAi-mediated gene-knockdown. A body of knowledge already exists, which allows the assessment of the success of screens. However, our understanding of the regulation of the pathway is incomplete, and this provides an opportunity for new discoveries to be made.

Screens can be carried out in a number of ways, using a range of different reagents. This work focuses on materials that are widely available, without recourse to specialist equipment or custom reagents. Although this may mean that better reagents and paradigms could have been constructed, ultimately the usefulness of these techniques as everyday tools for examining gene function relies on the use of standard, widely available resources.

Thus the aims of this thesis are:

1. To assess different methods for carrying out genome-scale RNAi screens using an assay for TRAIL-induced apoptosis.
2. To carry out screens to identify new genes in the TRAIL-induced apoptosis pathway and rigorously confirm hits for their reproducibility and specificity.
3. To assess the success and usefulness of the screens and the methodologies with which they were carried out for elucidating gene function.

2 METHODS

The methods presented here are in one of two formats. Format one is step by step instructions for each of standard protocols used. Format two is a description of how these protocols were applied to complete each experiment. Unless otherwise stated all reagents were from Sigma. .

2.1 Common Solutions and Media

2.1.1 LB Broth

10g Tryptone
5g Yeast extract
10g Sodium chloride
Upto 1l Double-distilled water
... pH to 7.0

2.1.2 2XLB Broth (low salt)

20g Peptone
10g Yeast Extract
5g Sodium chloride
Upto 1l Double-distilled water

2.1.3 Phosphate Buffered Saline (PBS)

36.65 g Sodium chloride
11.80 g Disodium hydrogen phosphate (Na_2HPO_4)
6.60 g Sodium dihydrogen phosphate (NaH_2PO_4)
up to 5 l Double-distilled water

2.1.4 Creosol Red Loading Buffer

28g Sucrose
100ml Filter sterilised T0.1E
8mg Creosol Red

2.1.5 T0.1E

10mM Tris ($\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3$)
0.1mM EDTA
.... pH 8

2.1.6 siRNA Suspension Buffer

11.78g Potassium Acetate
2.56g HEPES-KOH
800 μ l 1M Magnesium Acetate

398.2µl HPLC grade Water
... pH to 7.4 with KOH

2.1.7 HeLa Growth Media

50ml Modified Eagle's Medium (Sigma #M2279)
5ml Fetal Bovine Serum (Gibco #10270-106)
0.5ml 200mM L-Glutamine
0.5mls 100 Uml⁻¹ Penicillin
5g Streptomycin
0.5ml 100x Non-essential amino acids (Gibo, # 11140-035)

HeLa Seed Media

50ml Modified Eagle's Medium (Sigma #M2279)
5ml Fetal Bovine Serum (Gibco #10270-106)
0.5ml 200mM L-Glutamine
0.5ml 100x Non-essential amino acids (Gibo, # 11140-035)

2.1.8 Treatment/Assay media

10ml Modified Eagle's Medium (Sigma #M2279)
100µl 200mM L-Glutamine
100µl 20 Uml⁻¹ Penicillin
1g Streptomycin
100µl 100 x Non-essential amino acids (Gibo, # 11140-035)

TRAIL/FAS/H₂O₂ was added as required to make treatment media. 5mls alamarBlue was added to 45mls treatment media to make alamarBlue assay media. .

2.2 Common cell culture methods

2.2.1 Routine Passage of cells

Cells were routinely grown in a Galaxy R incubator (Scientific Laboratory Supplies) at 37°C, 5% CO₂ in 75 cm² culture flasks with 0.2µm vent caps (Corning #430641). Cells were passaged every 3 or 4 days. All solutions are warmed to 37°C. Growth media was made fresh each time cells were passaged.

1. Media was aspirated using a disposable glass pipette attached to a vacuum trap in 2% virkon.
2. Cells were washed with approximately 20mls of PBS, PBS was aspirated off.

3. Cells were washed with 3mls of 1XTypsin-EDTA solution and wash aspirated off.
4. Cells were washed with a further 3mls of 1XTypsin-EDTA solution and the wash decanted into 1% virkon.
5. Flask was incubated for 5 minutes at 37°C.
6. 10mls of HeLa growth media was added to the flask and cells suspended by pipetting up and down.
7. Cells were counted using an Improved Neubauer Haemocytometer (Assistant #403002400).
8. Cells were diluted to 1×10^5 cellsml⁻¹ in 20mls of growth media per new flask to be seeded.
9. 20mls of diluted cells were added to each new flask required. .

2.2.2 Defrosting of cells

All solutions were warmed at 37°C before use. Cells were stored in Liquid Nitrogen in Fetal Bovine Serum plus 10% DMSO.

1. 10mls of Growth Media was added to a T25 0.2µm vented flask (Corning #430639) and the flask marked passage 0.
2. Cells were defrosted in 37°C water bath.
3. A plastic pasteur pipette was used to add 1ml growth media to the cells.
4. Cells were transferred to flask containing media.
5. Cells were incubated overnight in 37°C, 5% CO₂ incubator.
6. Cells were passaged as described above (section 2.2.1).

2.2.3 RNA transfection of cells

Media was warmed to 37°C before use and transfection reagent was warmed to room temperature.

1. 2.5µmol per well of the siRNA(s) to be transfected was arrayed in a 96 well, round bottomed plate allowing one extra well per siRNA excess. .
2. 0.12µl of Lipofectamine 2000 (Invitrogen #11668-019) was diluted in 12.5µl of Opti-MEM I (Invitrogen #31985-047) per well to be transfected plus 10% excess and incubated for 15 minutes.
3. siRNA were diluted in 12.5µl Opti-MEM I per well to be transfected .
4. 12.5µl of Lipofectamine 2000/Opti-MEM I mixture per well to be transfected was added to the diluted siRNAs and incubated for a further 15 minutes. .
5. 25µl of siRNA/Lipofectamine 2000/Opti-MEM I mixture was added to the well of a

96 well plate containing cells seeded 24 hours earlier.

This protocol is for transfecting cells with 2.5pmol of siRNA in a 96 well plate. Unless otherwise stated this is how cells were transfected with siRNA. In other cases differing amounts of siRNA were used as specified in the text. Where cells were transfected in 24 well plates, 12.5µl of siRNA was diluted in 50µl Opti-MEM I and 0.6µl of Lipofectamine was mixed with 50µl Opti-MEM I.

2.2.4 Transfection of plasmid DNA

Media was warmed to 37°C before use. siPort XP-1 was warmed to room temperature before use.

1. 80ng of plasmid DNA per well to be transfected was aliquoted into the well of a round bottomed 96 well plate allowing one well extra excess for each different plasmid.
2. 0.24µl of siPort XP-1 per well to be transfected, plus 10% was diluted in 20µl Opti-MEM I and incubated for 10 minutes.
3. 20µl of siPort XP-1/Opti-MEM I per well to be transfected was added to plasmid DNA and incubated for a further 10 minutes.
4. 20µl of DNA/Opti-MEM I/siPort XP-1 mixture was added to each well of cells to be transfected.

The above protocol is for the transfection of 80ng of plasmid DNA using 0.24µl siPort XP-1 into cell grown in 96 well plates. Unless otherwise stated in the text this is how DNA transfection were performed. In some cases different quantities of DNA and siPort XP-1 were used as indicated in the text. Where cells were transfected in 24 well plates, 400ng of DNA was used per well and 1.2µl of siPort XP-1 was diluted in 100µl of Opti-MEM I.

2.2.4.1 Transfection Protocol using GeneJuice

Media was warmed to 37°C, and the GeneJuice reagent to room temperature before beginning. Protocol presented is for transfection in 24 well plates/8-well slides.

1. 200ng per well of DNA to be transfected was aliquoted into the well of a round bottomed 96 well plate, allowing one well extra excess.
2. 0.75µl GeneJuice (Novagen #70967-3) per well was mixed with 20µl Opti-MEM I per well, allowing one well excess, and incubated for 10 minutes at room temperature.
3. GeneJuice/Opti-MEM I mixture was added to plasmid DNA and incubated for 10

minutes.

4. 20µl GeneJuice/Opti-MEM I/DNA mixture was added to each well to be transfected. .

2.2.4.2 Transfection Protocol using Lipofectamine 2000

Media was warmed to 37°C and Lipofectamine 2000 reagent to room temperature before beginning. Protocol presented is for transfection in 24 well plates/8-well slides.

1. 1µg per well of plasmid DNA was aliquoted into a well of a round bottomed 96 well plate, allowing one well excess.
2. 1.5µl per well of Lipofectamine 2000 was added to 50µl per well of Opti-MEM I, allowing one well excess and incubated for 5 minutes.
3. 50µl per well of OptiMEM I was added to plasmid DNA, allowing one well excess.
4. 50µl per well of Lipofectamine 2000/OptiMEM I mixture was added to DNA/OptiMEM I mixture, allowing one well excess, and incubated for 20 minutes.
5. 100µl of Lipofectamine 2000/plasmid DNA/OptiMEM I mixture was added drop-wise to each well of cells and mixed by rocking.

2.2.4.3 Transfection Protocol using Effectene

Effectene used directly from the 4°C, but was not kept on ice during procedure. Buffers mentioned come from the Effectene Transfection Kit (Qiagen #301425). Protocol presented is for transfection in 24 well plates/8-well slides. .

1. 0.5µl per well of plasmid DNA was aliquoted into a well of a round bottomed 96-well plate and diluted to 146µl per well in Buffer EC. 4µl per well of Enhancer reagent was added.
2. Mixture was incubated at room temperature for 5 minutes.
3. 5µl per well Effectene reagent was added to 95µl per well of Buffer EC and this mixture added to the DNA/Enhancer Mixture and mixed by pipetting up and down 5 times.
4. Mixture was incubated for 10 minutes at room temperature.
5. 250µl of Effectene/DNA/Enhancer was added to each well of cells.

2.2.5 Isolation of total cellular RNA

Unless otherwise specified reagents are from the SV Total RNA isolation kit (Promega #Z3100). After lysis, lysates were stored as -70°C until purification. Samples were generally

processed in batches of 12.

1. 300µl of RNA Lysis Buffer was added to each well of cells .
2. Cells were left to lyse for 5 minutes on ice.
3. Plates were sealed with Parafilm (Fisher #SEL-400-050J) and stored at -70°C.
4. Plates were defrosted at room temperature.
5. Lysates were transferred to a 1.5ml tube.
6. Lysates were incubated at 70°C for 3 minutes in a water bath.
7. Lysates were centrifuged at 14,000g for 10 minutes.
8. Cleared lysates were transferred to a fresh 1.5ml tube.
9. 200µl 95% Ethanol was added to lysate and mixed by pipetting 3-4 times.
10. Half of lysate/ethanol mixture was transferred to SV RNA columns.
11. Columns were centrifuged at 14,000g for 1 minute.
12. Steps 10 and 11 were repeated for second half of lysate and elute discarded.
13. 600µl of SV RNA wash solution was added to each column.
14. Columns were centrifuged at 14,000g for 1 minute and elute discarded.
15. DNase mix was made up as the following master mix:

	1X	12x
0.009M MnCl ₂	5µl	60µl
SV Yellow Core Bufer	40µl	480µl
SV DNase	5µl	60µl

and 50µl added to each column.

16. Columns were incubated at room temperature for 15 minutes.
17. 200µl of SV DNase stop solution was added to each column.
18. Columns were centrifuged at 14,000g for 1 minute.
19. 600µl SV RNA wash solution was added to each column.
20. Columns were centrifuged at 14,000g for 1 minute and elute discarded.
21. 250µl SV RNA wash solution was added to each column.
22. Columns were centrifuged at 14,000g for 2 minutes and collection tube discarded.
23. Cap was removed from the columns and the columns were placed in elution tubes.
24. 100µl nuclease water was added to each column and the columns were centrifuged for 1 minute at 14,000g.
25. 2µl of 5M NaCl and 250µl ice-cold 100% Ethanol was added to each elute.
26. Elute was incubated for 1 hour at -20°C.
27. RNA was collected by centrifugation at 14,000g for 30 minutes at 4°C.
28. Supernatant was carefully removed by pipetting.

29. Pellet was air-dried for 5 minutes and dissolved in 13.2µl Nuclease free water .

2.2.6 Preparation of samples for determination of mRNA knock-down by qRT-PCR

2.2.6.1 siRNA mediated knock-down

1. Passage 3 cells were passaged as described in 2.2.1 and resuspended in HeLa seed media.
2. Cells were diluted to 3×10^4 cellsml⁻¹ in HeLa seed media and 0.5mls seed into enough wells of a 24 well cell culture cluster (Corning #3524) to allow one well per siRNA, plus one extra. Cell were allowed to adhere to the plate for 15 minutes and then incubated at 37°C, 5% CO₂ for 24 hours.
3. Cells were transfected with 12.5pmol of each siRNA plus 12.5pmol of siNeg control.
4. Cells were incubated at 37°C, 5% CO₂ for 24 hours.
5. Media was removed by aspiration and replaced with HeLa growth media and cells were grown for a further 24 hours.
6. Media was aspirated and total cellular RNA isolated as described above (2.2.5).

2.2.6.2 shRNA mediated knock-down

1. Passage 3 cells were passaged as described in 2.2.1 and resuspended in HeLa seed media.
2. Cells were diluted to 1.2×10^5 in HeLa seed media and 0.5ml seeded into enough wells of a 24 well cell culture cluster to allow one well per shRNA encoding plasmid, plus one extra. Cells were allowed to adhere to the plate for 15 minutes and then incubated at 37°C, 5% CO₂ for 24 hours.
3. Cells were transfected with 200ng of each shRNA encoding plasmid plus pSM2.shControl and 200ng pIRES-P plasmid.
4. Cells were incubated at 37°C, 5% CO₂ for 24 hours.
5. Media was removed by aspiration and replaced with 0.5ml HeLa growth media plus 2µg/ml puromycin. Cells were incubated at 37°C, 5% CO₂ for a further 48 hours.
6. Media was removed by aspiration and total cellular RNA isolated as described above (2.2.5).

2.2.7 Screening Methods

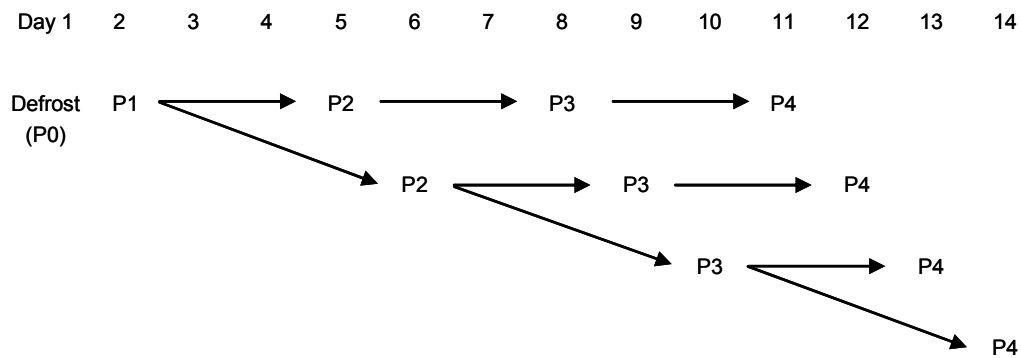


Figure 2.1 Scheme for growing cells for use in screening

Scheme ensures that cells, passaged four times since removal from liquid nitrogen storage, are available on four consecutive days. Cells are passaged every 3 or 4 days. Flasks passaged after four days are split into two flasks, with one flask passaged after 3 days and the other after 4 days. P - Passage

2.2.7.1 Seeding Cells for Library Transfection

1. Cells were trypsinized as described (2.2.1) and resuspended in 10ml Seed Media.
2. Cells were counted and sufficient cells for 10mls per plate diluted to 3×10^4 cell/ml

$$cells(ml) = \frac{3 \times 10^4 \text{ cells / ml} \times 10ml \times plates}{cell_density}$$

3. A 1250 μ l electronic repeating multi-channel pipette (Matrix Impact 1250, Thermo Scientific) was used to add 100 μ l of cell suspension to the first 11 columns of each plate, and 6 of the 8 wells of the 12th column of a 96 well OptiLux white walled tissue culture plate (BD Bioscience #353947).
4. Plates were left on bench for 20-30 minutes.
5. Plates were placed in a polystyrene box containing sterile water and the lid sealed, with one corner unsealed (semi-sealed).

2.2.7.2 Transfection of Library siRNAs

Aliquots of original library plates were diluted to 1.25 μ M in siRNA suspension buffer and stored at -20°C. Sufficient control siRNAs for 100 wells were diluted to 1.25 μ M in siRNA and arrayed in a 96 well plate in layout used in screen (see Figure 4.1).

The following protocol is based on 12 plates. If more plates than this were processed in a day, the protocol was repeated.

1. Diluted library plates plus the plate contain controls were removed from freezer and defrosted on bench. .

2. Plates were centrifuged at 3,000 rpm for 5 minutes in a bench top centrifuge (Sorvall RT7).
3. 2µl of each siRNA (1.25µM) was transferred to a labelled V bottomed plate (VWR # 732-2702) excluding wells to contain controls. .
4. 2µl of control siRNAs added to 2 empty columns on each plate.
5. 13.75µl per plate Lipofectamine 2000 was diluted in 1.43ml per plate OptiMEM I , for first 6 plates and incubated for 15mins.
6. 13µl OptiMEM I was added to each well of the siRNA contain plates using 125µl electronic repeating pipette (Matrix Impact2 125, Thermo Scientific) .
7. Step 5 was repeated for second 6 plates.
8. Lipofectamine 2000/OptiMEM I mixture from step 5 was added to first six siRNA containing plates and the mixture was incubated for 15 minutes.
9. Plates containing cells were checked for contamination. .
10. Lipofectamine 2000/OptiMEM from step 7 was added to second six siRNA containing plates and mixture incubated for 15 minutes.
11. Lipofectamine 2000/OptiMEM I/siRNA mixture from step 8 was added to wells of cell containing plates and mixed by gentle pipetting.
12. Step 11 repeated for mixture from step 10.
13. Cells were returned to 37°C, 5% CO₂ incubator for 24 hours.
14. Media was removed using 8-channel aspirator and replaced with 100µl HeLa growth media using electronic repeating pipette.

2.2.7.3 Transfection of library DNA

Library was provided by the Chromosome 22 ORF team at the Wellcome Trust Sanger Insitute as six plates of DNA prepared using Qiagen QIASpin miniprep kits at a concentration of 50µg/ml. The following protocol is for three plates. If more plates were required, the protocol was repeated.

1. Construct containing plates were defrosted on bench.
2. Construct containing plates were spun down at 3000rpm for 5 minutes in a benchtop centrifuge (Sorvall RT7).
3. 1.6µl (80ng) of DNA was transferred from each well of the library plate to a fresh 'V-bottom' 96 well plate (VWR # 732-2702).
4. 26.4µl per plate siPort XP-1 (Ambion # AM4507) was mixed with 2.2ml per plate OptiMEM I and incubated for 10 minutes at room temperatue.

5. 20µl siPort XP-1/OptiMEM I mixture was added to each well of plasmid DNA. .
6. Complex/DNA was collected in the base of the well and mixed by gently tapping the plate and incubated for 10 minutes at room temperature.
7. Complex/DNA was transferred to plates containing pre-plated cells and mixed by gentle pipetting.
8. Cells were returned to 37°C, 5% CO₂ incubator for 24 hours.
9. Media was removed using 8-channel aspirator and replaced with 100µl HeLa growth media using electronic repeating pipette. .

2.2.7.4 alamarBlue Assay

The protocol here is based on 12 plates. If more plates were to be assayed, the protocol was repeated as necessary. Sufficient assay media was made to assay each plate twice immediately prior to pre-treatment reading.

1. Media was aspirated from wells using 8-channel aspirator and replaced with 100µl alamarBlue assay media.
2. Plates were incubated at 37°C, 5% CO₂ for 3 hours.
3. Condensation was removed from inside of plate lid using a tissue immediately before reading.
4. Level of fluorescence was detected by plate reader with the following settings:

Excitation wavelength: 544nm.

Emission wavelength: 590nm.

Reads: 6.

Temperature: 37°C.

2.3 Common molecular biology methods

2.3.1 Transformation of plasmid DNA into *E.coli*

1. 0.5µl of each DNA to be transformed was aliquoted into the well of a 96 well PCR plate, plus one well containing 0.5µl 1ng/µl pUC19 DNA as a positive control.
2. Plate was spun down briefly.
3. Plate was incubated at -80°C to allow DNA to freeze.
4. Competent cells were thawed for 5 minutes on a metal cold block (Stratagene).
5. Competent cells were mixed by gently flicking the tube.
6. Plate was placed on a metal cold block inside a benchtop cooler (StrataCooler) to

maintain plate at 4°C.

7. 10µl of cells was added to each well of the plate containing DNA.
8. Plate was incubated at 4°C for 20 minutes.
9. Cells were heat shocked at 42°C for 45 seconds in a thermocycler (MJ, PTC-225).
10. Cells were incubated for 2 minutes at 4°C.
11. 90µl of ice cold LB broth was added to each well.
12. Cells were incubated for 90 minutes at 37°C.
13. Cells were spread onto LB agar plates containing appropriate antibiotic to select for transformants. The pUC19 sample was spread on LB agar plates containing 100µg/ml ampicillin.
14. Plates were grown over night at 37°C, colonies counted and stored at 4°C until needed. .

2.3.2 Digestion with Restriction Enzymes

Protocol presented here is for a 100µl reaction. Reactions of different sizes were scaled directly from this protocol except for quantity of enzyme(s) which was always 1µl unless otherwise stated.

1. Reaction pre-mix sufficient for each reaction required, plus one extra was made up

<i>Reagent</i>	<i>1X</i>
10x Buffer	10µl
Creosol Red Loading Buffer	34µl
Bovine Serum Albumen	1µl
Enzyme 1	1µl
(optional) Enzyme 2	1µl
Double distilled water	.. upto (100-x)µl.

Where x is the volume of DNA to be added.

2. DNA is added to pre-mix and incubated for 1-16 hours at 37°C.
3. Reactions were terminated by incubating reaction at 80°C for 15 minutes.

2.3.3 Agarose Gel electrophoresis

Strength and size of gel is specified in text.

1. Gel was prepared in 50mls (mini-gel) or 250mls (maxi-gel) 1XTBE containing 250ng/ml ethidium bromide and appropriate quantity of agarose (Invitrogen #15510027).
2. Samples were run in 30% Creosol Red loading buffer at either 80v for 20minutes (mini-gel) or 200v for 1 hour (maxi-gel).
3. Products were visualised using UV illumination and recorded using a digital gel

documentation system (UVP #GelDoc-IT).

2.3.4 KOD Polymerase Chain Reaction (KOD PCR)

Unless otherwise stated in text, PCR reactions carried out were done so with KOD Hot Start Polymerase (Novagen #71086-3) using the following protocol.

1. A PCR reaction premix sufficient for the number of reactions to be carried out plus one was made up

Reagent	1Xl
10x KOD Buffer	2.5µl
2mM dNTPs	2.5µl
25mM MgSO ₄	1µl
Creosol Red loading buffer	8.3µl
Double distilled water	3.7µl
KOD polymerase (1 Uµl ⁻¹)	1µl
Forward Primer (15µM)*	0.5µl
Reverse Primer (15µM)*	0.5µl

* Primers were only included in master mix if all reactions required the same primers.

2. Mix was briefly vortexed and spun down.
3. 20µl of premix was added to wells of a 96 well PCR plate (ABgene #AB800).
4. 5µl template diluted in double distilled water was added to each well.
5. PCR was carried out using the following program on a 96 well thermocycler (MJ #PTC-225).
 - 95°C for 2 minutes.
 - 95°C for 20 seconds, 60°C for 30 seconds, 70°C for 1 minutes - 30 cycles.
 - 68°C for 5 minutes.
 - 10°C forever.

2.3.5 Quantitative Polymerase Chain Reaction (qPCR)

All reactions were carried out in triplicate. Pre-mixes were made up for the number of reactions required plus 10%. Reactions were performed using reagents from the SYBR green qPCR MasterMix Plus kit (Eurogentec # RT-QP2X-03-075+).

1. PCR pre-mix was diluted with double distilled water:

<i>Reagent</i>	<i>1X/µl</i>
2x qPCR MasterMix	12.5µl
Double distilled water	6.5µl
Primer Mix (7.5µM)*	2µl

* Primer mix was only added if only one primer was used.

2. 19 μ l (without primer) or 21 μ l (with primer) of pre-mix was added to 96 well optical reaction plate (ABI # N8010560) using a multichannel pipette.
3. If primers not already added 2 μ l primer was added to each well.
4. 4 μ l template was added to each well.
5. Plate sealed with an optical plate seal (ABI #4311971).
6. Plate spun at 3000 rpm for 3 minutes in a bench top centrifuge (Eppendorf 5403) and inspected to ensure no wells contained bubbles.
7. PCR reactions carried out on either an ABI7000 or ABI7900 sequence detection systems using the following program:
 - 50°C for 2 minutes.
 - 95°C for 10 minutes.
 - 95°C for 15 seconds, 60°C for 1 minute, 40 cycles*.
 - Melt Curve: 95°C for 15 seconds, 60°C for 15 seconds, 95°C for 15 seconds, ramping at 2% of maximal speed*.

* data was collected during these stages.

2.3.6 Reverse Transcription

All reagents are defrosted in metal cold blocks cooled to 4°C. Work is carried out in an RNase free environment with RNase free solutions and pipettes. Unless otherwise stated reagents come from Superscript II Reverse Transcription Kit (Invitrogen # 18064-071).

1. 11 μ l of each RNA was aliquoted into a 96 well PCR plate (ABgene #AB800).
2. 1 μ l 12.5 μ g/ μ l oligo dT (Invitrogen #18418012) was added to each well.
3. Plate was heated to 70°C for 10 minutes and then rapidly cooled to 42°C in a 96 well thermocycler (MJ, PTC-225).
4. To each well the following was added

<i>Reagent</i>	<i>1X/μl</i>
5x First Strand Buffer	4 μ l
0.1 M DTT	2 μ l
10 mM dNTP mix (Invitrogen, #18427013)	1 μ l

5. Plate was heated to 42°C for 2 minutes.
6. 1 μ l Superscript II reverse transcriptase was added to each well.

7. Plate was incubated at 42°C for 50 minutes.
8. Enzyme was inactivated by heating at 70°C for 15 minutes.

2.3.7 Measuring efficiency of qRT-PCR oligonucleotide primers

1. cDNA was prepared from HeLa cells by extracting total cellular RNA from 5×10^6 HeLa cells and Reverse Transcription of 5µg as described in 2.2.5 and 2.3.6.
2. KOD PCR was performed on 25ng HeLa cDNA as a template using the primer being tested. Products were analysed by agarose gel electrophoresis to ensure one and only one product of the correct size was present.
3. Dilutions of HeLa cDNA were prepared to allow 3µl of 25ng/µl, 5ng/µl, 1ng/µl and 0.2ng/µl cDNA per primer to be tested plus 3µl excess, by serial dilution.
4. Diluted HeLa cDNA was used as a template to perform SYBR green qPCR. Three reactions were run with each dilution of the template for each primer to be tested, plus one reaction for each primer using double distilled water as a template.
5. Melt-curves were examined to ensure the absence of primer-dimers.
6. Primer efficiencies were calculated using the qBase software.

2.3.8 Measurement of RNAi mediated mRNA knock-down by qRT-PCR

1. RNA samples were prepared as described above (2.2.6).
2. RNA samples were reverse transcribed into cDNA (2.3.6).
3. Success of reverse transcription was confirmed by KOD PCR with 1µl of each sample as template using primers designed to amplify a section of the ARSA gene and products analysed by agarose gel electrophoresis on a 2% gel.
4. Samples were diluted 1:10 with double distilled water, except negative controls which were diluted 1:20.
5. 4µl of each sample was used in qPCR for the following genes: the gene of interest, GAPDH and ACTB in triplicate as described in 2.3.5.
6. 4µl of negative control sample was used in qPCR for all genes of interest plus GAPDH and ACTB.
7. Where possible all reactions involving a gene were kept on the same plate.
8. Ct values were calculated using an automatic baseline and a threshold of 0.2 in the ABI SDS software.
9. Data was analysed using the qBase software (Hellemans et al. 2007): Replicates more

than 0.5 Ct from other replicates were excluded from analysis. GAPDH and ACTB were used as normalization standards and all results are presented relative to the negative sample. .

2.4 Computational methods

2.4.1 Design of oligonucleotide primers for qRT-PCR

Where possible oligonucleotide primer sequences were taken from RTPrimerDB (Pattyn et al. 2006) or existing publication as specified in Appendix B. .

1. Sequence and exon/introns boundaries for transcript was extracted from the Ensembl human database (www.ensembl.org, release 45 at time of writing).
2. Primers were designed using the Primer3 software (Rozen, Skaletsky 2000) using the default setting except:

<i>Setting</i>	<i>Value</i>
Max Tm difference	1
Max self complementarity	4.00
Product Size Ranges	50-100 150-250

3. Primers were designed so that either the amplicon included an introns of at least 5kb or one of the oligonucleotides contained sequence from two exons.
4. Specificity of primers was checked using the In-silico PCR tool at <http://genome.brc.mcw.edu/cgi-bin/hgPcr>.

2.4.2 Analysis of screening data

The files output from the plate reader are in the incorrect format to be processed by the R/Bioconductor package cellHTS. A Perl script was used to subtract the background fluorescence from each value and reformat the plate reader output file so that they could be read by cellHTS.

Screening data was analysed in the R/Bioconductor package cellHTS using scripts available in description file that is attached to the HTML reports for each screen, available in the appropriate appendix (on the included CD or on-line).

Briefly, each script:.

1. Loads plate data into a single cellHTS object x.
2. Loads plate layouts, screen description and log files.
3. Removes values of blank wells and siKIF11 wells.
4. (for Druggable genome and chromosome 22 screens only) Normalizes each plate in each channel to the plate median and removes wells in channel 1 which are below a

threshold.

5. Calculates survival percentages, and normalizes these survivals to the plate median and log transforms data (Druggable genome screen and Chromosome 22 screen).
6. Calculates scores for each “probe” by calculating z-scores and selecting the minimum of the two replicates.
7. Loads gene annotation information.
8. Outputs a HTML report.
9. Calculates the results summarised by gene.

Mean/Sd plots were generated using the R/Bioconductor package vsn’s function meanSd.

2.4.3 Analysis of seed sequences

siRNA sequences for siRNAs in the library are provided as the sequences of the target, which is equivalent to the sense strand of the siRNA. Seed sequences are therefore bases 15-20 or 14-20 of this sequence.

2.4.3.1 Assessment of significance of ‘hit’ seeds

The hexamer and heptamer seed sequences of the siRNAs in the Qiagen Druggable genome V2 library were extracted and read into R. The sample and replicate functions were used repeatedly select 20 seed sequences at random from the list of siRNA seed sequences. The table function was used to count the appearances of each seed in each sample. P values were calculated by counting the number of samples in which x seeds occurred more than y times and dividing by the total number of samples. For calculations of the hexamer seeds 5,000 samples were used. For calculations of the heptamer seeds only 2,000 samples were used due to memory restraints. .

2.4.3.2 GSEA of ranked seeds

A ranked list of siRNA sequences was extracted from the screen results along with the score the siRNA obtained. The hexamer/heptamer for each siRNA was determined. siRNAs were placed into sets depending on the seed and formatted for the GSEA-P software using a Perl script.

See section 0 for details of GSEA. GSEA preranked analysis was performed using 1,000 repetitions, using the ranked list of siRNAs as the ranked list and the seed sequence sets for the gene set database. All other parameters were set to default. Seed sequences were said to

be significant if $FWER < 0.05$.

2.4.3.3 Assessing average frequency of seed matches in 3'UTRs

The set of human 3'UTRs from ensembl was retrieved using the BioMart data mining software. The average frequency of each possible 6nt, 7nt and 8nt sequence in each 3' UTR and also the frequency of matches in total 3' UTR sequence was calculated using the perl script, written by Dr. A. Enright using the following formulas:

$$\overline{Frequency}_s = \frac{\sum_n^N \frac{count(s, utr_n)}{length(utr_n)}}{N}$$

$$Bulk_Frequency_s = \frac{\sum_n^N count(s, utr_n)}{\sum_n^N length(utr_n)}$$

where s is some sequence, N is the number of UTRs in the set, and utr_n is the n th UTR in that set. $count(s, utr_n)$ is the number of occurrences of s in utr_n and $length(utr_n)$ is the length of utr_n in kilobases. $count(s, utr_n)$ was implemented using a regular expression search, implemented with the code snippet:

```
$matches = () =~ ($utrseq =~ /$seedseq/g) .
```

This script takes as its input a set of UTRs and a length donating the length of sequence to be searched for. It then generates all possible sequences of that length, and calculates their average frequency in the set of UTRs and also their bulk frequency as described above. It outputs a table of sequences follow by each of the different measures of frequency. This output was analysed and plotted in R.

2.4.3.4 Searching for possible transcripts with matches to hit and enriched seeds

A Perl script was implemented which takes a set of 3' UTR sequences and a list of seeds and outputs the number of seeds found in each of the 3' UTRs and the frequency with which matches are found:

$$MatchFrequency_{s, utr} = \frac{\sum_{s \in S} count(s, utr)}{length(utr)}$$

where S is the set of seeds being examined, utr is some utr being examined and $count$ and $length$ are as described above.

A second Perl script takes a list of gene names, a list of seed sequences and a list of 3'UTRs and outputs a list of genes from gene list that contain a match for each of the seeds in the seed list. .

2.5 Chapter 3 methods

2.5.1 TRAIL cytotoxicity assays

Many of the parameters in this protocol were changed during the optimisation process. Presented here is a template protocol, the values of various parameter used is specified in the text when non-standard.

2.5.1.1 Treated vs. Untreated assay

1. Passage 3 cells were passaged (see 2.2.1) and resuspended in HeLa Seed Media.
2. Cells were diluted to 3×10^4 cellsml⁻¹ in HeLa Seed Media.
3. 100µl of cells were seeded into a 96 well OptiLux white walled tissue culture plate (BD Bioscience #353947).
4. Two wells were left empty for blanking measurements.
5. Plates were placed in a semi-sealed polystyrene box stood on petri-dishes of sterile water.
6. Cells were allowed to adhere to plate for 15 minutes at room temperature.
7. Cells were incubated at 37°C, 5% CO₂ for 24 hours.
8. (optional) Cells were transfected with amount of siRNA or plasmid DNA indicated (see 2.2.3 and 2.2.4 respectively).
9. Cells were incubated for 48 hours at 37°C for 24 hours.
10. Media was removed by pipetting with a multi-channel pipette and discarded into 1% virkon.
11. 100µl TRAIL treatment media containing appropriate concentration of TRAIL and serum was added half of the wells.
12. 100µl TRAIL treatment media containing no TRAIL and appropriate concentration of serum was added to the remaining wells.
13. Plates were incubated for 24 hours.
14. Media was removed by pipetting with a multi-channel pipette and discarded into 1% virkon.
15. 100µl alamarBlue assay media was added to each well.
16. Plates were incubated at 37°C, 5% CO₂ for 3 hours.

17. Plates were read in a Gemini SpectraMAX plate reader (Molecular Devices #XPS) using the following settings:
 - Temperature: 37°C.
 - Excitation Wavelength: 544nm.
 - Emission Wavelength: 590nm.
 - Accuracy: 10 readings.
18. Average value of fluorescence in the blank wells was subtracted from the fluorescent reading in all other wells.
19. Survival for each condition was calculated: $survival = \frac{\bar{f}l_{treated}}{\bar{f}l_{untreated}} \times 100\%$ where $\bar{f}l_{treated}$ and $\bar{f}l_{untreated}$ are the average fluorescence for treated and untreated wells for a particular condition or transfected with a particular construct. .

2.5.1.2 Before vs. After assay

1. Passage 3 cells were passaged (see 2.2.1) and resuspended in HeLa Seed Media.
2. Cells were diluted to 3×10^4 cellsml⁻¹ in HeLa Seed Media.
3. 100µl of cells were seeded into a 96 well OptiLux white walled tissue culture plate (BD Bioscience #353947).
4. Two wells were left empty for blanking measurements.
5. Plates were placed in a semi-sealed polystyrene box stood on petri-dishes of sterile water.
6. Cells were allowed to adhere to plate for 15 minutes at room temperature.
7. Cells were incubated at 37°C, 5% CO₂ for 24 hours.
8. (optional) Cells were transfected with amount of siRNA or plasmid DNA indicated (see 2.2.3 and 2.2.4 respectively).
9. Cells were incubated for 24 hours at 37°C for 24 hours.
10. If cells were transfected, media was removed by pipetting with a multi-channel pipette and discarded into 1% virkon and replaced with 100µl HeLa growth media.
11. Cells were incubated for 24 hours at 37°C.
12. Media was removed by pipetting with a multi-channel pipette and discarded into 1% virkon and replaced with 100µl alamarBlue assay media.
13. Plate was incubated for 3 hours.
14. Plates were read in a Gemini SpectraMAX plate reader (Molecular Devices #XPS)

using the following settings:

Temperature: 37°C.

Excitation Wavelength: 544nm.

Emission Wavelength: 590nm.

Accuracy: 10 readings.

15. Media was removed by pipetting with a multi-channel pipette and replaced with 100µl TRAIL treatment media with appropriate concentration of TRAIL ligand.

16. Plates were incubated for 20 hours at 37°C, 5% CO₂.

17. Steps 10-12 repeated.

18. Average value of fluorescence in blank wells was subtracted from the fluorescence values in all other wells.

19. Survival of cells in each well was calculated: $survival = \frac{fl_{after}}{fl_{before}} \times 100\%$ where

fl_{after} and fl_{before} are the fluorescence readings for that well after and before treatment respectively. .

2.5.2 Assessment of plasmid transfection efficiency

1. Passage 3 cells were passaged as described in 2.2.1 and resuspended in HeLa seed media.

2. Cells were diluted to 5×10^4 cellsml⁻¹ and 0.5ml seeded into each well of an 8-well tissue culture slide (BD Falcon #354118), allowed to adhere for 15 minutes and incubated at 37°C, 5% CO₂ for 24 hours.

3. Cells were transfected with the pEGFP-N1 plasmid using the indicated transfection reagent as described in 2.2.4.

4. Cells were grown for 48 hours.

5. Cells were fixed by washing once with PBS and then incubating for 15 minutes in 3.7% Paraformaldehyde:

370mg	Paraformaldehyde
400mg	Sucrose
10mls	PBS
A few drops	1M Sodium Chloride

6. Cells were washed three times in PBS and the well divider removed.
7. Slide was dried and coverslip mounted using Vectashield + DAPI (Vector Laboratories #H-1200).
8. Slides were visualised on a fluorescent light microscope (Zeis Axioplan 2 microscope fitted with a Hamamatsu ORCA-ER camera) and images of three random fields of view captured from each well using both FITC and DAPI filters using the SmartCapture X software (Digital Scientific). .
9. Number of green fluorescent cells (number of transfected cells) and number blue fluorescent DAPI stained nuclei (total number of cells) was counted in each field of view and used to calculate transfection efficiency.

For experiments involving selection, cells were transfected with 100ng pEGFP-N1 and 100ng pIRES-P vectors using siPort XP-1. Cells were grown for 24 hours and the media changed for HeLa growth media containing 2µg/ml puromycin. Cells were grown for a further 48 hours before fixation. .

2.5.3 Cloning of hairpins targeting Caspase 8

Sequences of hairpin oligonucleotides were designed using the shRNA retriever tool <http://katahdin.cshl.org:9331/RNAi/html/rnai.html>. Sequences of these oligonucleotide templates were:

```
shCasp8.1  TGCTGTTGACAGTGAGCGAGGATACTGTCTGATCATCAACTAGTGAAG
           CCACAGATGTAGTTGATGATCAGACAGTATCCCTGCCTACTGCCTCGGA

shCasp8.2  TGCTGTTGACAGTGAGCGCTCTCGGACTCTCCAAGAGAACTAGTGAAG
           CCACAGATGTAGTTCTCTTGGAGAGTCCGAGATTGCCTACTGCCTCGGA

shCasp8.3  TGCTGTTGACAGTGAGCGCTGCCTTGATGTTATTCCAGAGAGTGAAGC
           CACAGATGTACTCTGGAATAACATCAAGGCATTGCCTACTGCCTCGGA
```

Sequence of mature siRNA sense strand shown in blue, antisense in bold.

1. 97bp hairpin oligonucleotide templates were amplified in a KOD PCR reaction using the primers 5'mirR30EcoRIA and 3'miR30XhoI:

```
5'mirR30EcoRIA  CAGAAGGCTCGAGAAGGTATATTTGCTGTTTACAGTGAGCG
3'miR30XhoI    CTAAAGTAGCCCCTTGAATTCCGAGGCAGTAGGCA
```

Using the following reaction pre-mix:

<i>Reagent</i>	<i>1X</i>
10x KOD Buffer	10 μ l
DMSO	5 μ l
Betane	5 μ l
2mM dNTPs	10 μ l
5' primer	1 μ l
3' primer	1 μ l
Template (100ng/ μ l)	1 μ l
25mM MgSO ₄	4 μ l
Double distilled water	70 μ l
KOD polymerase (1 U μ l ⁻¹)	2 μ l

Each reaction was set up in a 96 well PCR plate (ABgene #AB800).

2. PCR reactions were carried out on a 96-well thermocycler (MJ, PTC-225) using the following program:

- 94°C for 1 minutes, 54°C for 30 seconds, 75°C for 1 minutes – 1 cycle.
- 94°C for 30 seconds, 54°C for 30 seconds, 75°C for 1 minutes – 23 cycles.
- 94°C for 30 seconds, 54°C for 30 seconds, 75°C for 11 minutes – 1 cycle.
- 4°C forever.

3. PCR products were purified by extracting once in 100 μ l of phenol:chloroform:isoamyl alcohol (25:24:1) and once in 100 μ l of chloroform:isoamyl alcohol (24:1).
4. Products were precipitated by addition of 250 μ l of 96% ethanol and 10 μ l 3M Sodium Acetate and incubation at -20°C for 30 minutes. Pellets were collected by centrifugation at 16,000 x g for 20 minutes and washed in 70% ethanol and centrifuged at 16,000 x g for 20 minutes and resuspended in 20 μ l 0.1M TrisCl.
5. An A-tailing reaction pre-mix was made up sufficient for A-tailing reactions:

Reagent	1X
NEB Buffer	1 μ l
1mM dATP	2 μ l
Taq Polymerase	1 μ l
Double Distilled Water	4 μ l

6. 3 μ l (approximately 1.5 μ g) of each PCR product was added to 7 μ l A-tail pre-mix and incubated at 70°C for 30 minutes.
7. Ligation pre-mix sufficient to ligate each product in the pGEM-T easy vector was made up using reagents from the Roche Rapid Ligase Kit (Roche #11 635 379 001):

<i>Reagent</i>	<i>1X/</i>
5x Dilution Buffer	2 μ l
Double distilled water	5.5 μ l
pGEM-Teasy vector (Promega #A1360)	0.5 μ l
2x Ligation Buffer	10 μ l
T4 DNA Ligase	1 μ l

8. 1µl of each A-tailed product was added to 19µl to ligation pre-mix and incubated for 10 minutes at room temperature.
9. 0.5µl of each ligation product was transformed into JM109 competent cells (Promega #L2001) and transformants plated on X-gal/AMP/LB agar plates:
 - 1 litre LB agar at 52°C
 - 160µl 500mg/ml X-gal (Fisher # BPE1615-100) in demethylformide
 - 2ml 25mg/ml Ampicillin
 - 5ml 0.1mM Isopropyl-β-d-Thiogalactopyranoside (IPTG)
10. 12 white colonies from each transformation were picked into 1.8ml of LB broth + 50µg/ml ampicillin and incubated overnight at 37°C in a 2ml 96 well plate (costar #3961).
11. 240µl 50% glycerol was added to each well. Plate stored at -70°C.
12. 100µl of each clone sent for sequencing by the in-house sequencing service with the primers:
 - Forward: GTAAAACGACGGCCAGT
 - Reverse: GGAAACAGCTATGACCATG.
13. Sequences were check for expected insert using the cross_match algorithm.
14. One clone for each hairpin was cultured overnight in 10mls of LB broth plus 50µg/ml ampicillin. Each culture was split in two and plasmid DNA prepared using the Qiaquick Spin Miniprep kit (Qiagen, #27104) as per manufacturer's instructions. Each plasmid was eluted in 50µl EB buffer supplied with kit.
15. 10µg Plasmid DNA was digested for 1 hour using XhoI (New England Biolabs # R0146S) and EcoRI (New England Biolabs #R0101L) restriction enzymes in 50µl 1xEcoRI Buffer (New England Biolabs #B0101S).
16. Restriction products were size separated using agarose gel electrophoresis on a 2% agarose gel. The fragment of the correct size (121bp) was excised and purified using the Qiagen QIAquick gel extraction kit (Qiagen, #28704) as per manufacturer's instructions.
17. Vector was prepared by digesting 3.5µg of pSM2 vector for 2 hours with EcoRI and XhoI restriction enzymes in 200µl of EcoRI buffer.
18. Digested vector was precipitated in 2 volumes of 96% ethanol and 0.1 volumes of 3M Sodium Acetate at 4°C for 30 minutes. Pellet was collected by centrifugation at 16,000 x g for 30 minutes. Pellet was washed in 70% ethanol and resuspended in 60µl 0.1M Tris-EDTA.
19. Digested vector was size selected by agarose gel electrophoresis on 1% gel, and the

band of the correct size (approx 7kb) was excised and purified using the Qiagen QIAquick gel extraction kit as per manufacturer's instructions.

20. Hairpin inserts were ligated into prepared pSM2 vector using the following mix:

<i>Reagent</i>	<i>1X</i>
5x Dilution Buffer	2µl
pSM2 vector	2µl
Insert	2µl
Double distilled water	3µl
2x Ligation Buffer	10µl
T4 DNA Ligase	1µl

21. Ligation reactions were incubated for 10 minutes at room temperature.

22. 0.5µl of ligation product was transformed into PIR1 chemically competent cells (Invitrogen #C1010). Transformants were plated on LB agar plates containing 50µg/ml chloramphenicol and 25µg/ml kanamycin and incubated overnight at 37°C.

23. 8 colonies from each transformation were picked into 1.8ml 2XLB (low salt) plus 50µg/ml chloramphenicol and 25µg/ml kanamycin in a 2ml 96 well plate and cultured overnight at 37°C.

24. 240µl of 50% glycerol was added to each well and culture stored at -70°C.

25. 100µl of each culture was sequenced by the in-house sequencing service using the primer: GTAACTTGAAAGTATTTCG.

26. Sequences were compared to expected insert sequences using the cross_match algorithm. .

2.5.4 Comparison of effect of siRNA and shRNA mediated knock-down of Caspase – 8

For siRNAs, 12 wells of HeLa cells were transfected with 2.5pmol siCasp8 and 12 with 2.5pmol siNeg and their sensitivity to TRAIL measured as described in 2.5.1.2. Assays for the effect of shRNAs were carried out on the same plate by transfecting 12 wells with 80ng pSM2.shCasp8.2 and 12 with 80ng pSM2.shControl and measuring their sensitivity to TRAIL as described in 2.5.1.2. Assays were carried out using 0µg/ml, 0.25µg/ml, 0.5µg/ml and 1µg/ml in triplicate. This procedure was repeated using 3 separate aliquots of cells defrosted and grown independently. .

The test for the effect of selection was carried out by transfecting 12 wells with 40ng pSM2.shCasp8.2 and 40ng pIRES-P and 12 wells with 40ng pSM2.shControl and 40ng pIRES-P and measuring their sensitivity to TRAIL as described in 2.5.1.2 except that 24 hours after transfection media was removed and replaced with 100µl HeLa growth media

plus 2µg/ml puromycin and grown for a further 48 hours before assaying for the effect of TRAIL.

2.5.5 Blind pseudo-screens

All DNA used was prepared using the Qiagen HiSpeed Maxi prep kit (Qiagen #12663) as per manufacturer's instructions. Sequences of siRNAs can be found in Appendix A. For siRNAs, siRNAs were arrayed in a 96 well plate by experimenter 1 such that 5-15 wells contained 2.5pmol of siCasp8 and the rest 2.5pmol of siNeg. Plate was passed to experimenter 2 who transfected siRNAs into cells and measured their sensitivity to TRAIL as described in 2.5.1.2, using 1µg/ml TRAIL. Data was processed by dividing survival value in all wells by median survival of all wells on plate and standardised by calculating a score, z , thus:

$$z = \frac{x - \mu}{\sigma}$$

shRNA experiment was carried out identically except cells were transfected with pSM2.shCasp8.2 or shControl.

Experiment using selection was carried out as described for shRNAs except cells were seeded at 12,000 cells per well, media was removed 24 hours after selection and replaced with 100µl HeLa growth media plus 2µg/ml puromycin and cells were incubated for a further 48 hours at 37°C, 5% CO₂ before the effect of TRAIL was measured.

2.5.6 Comparison of siRNA mediated and shRNA mediated knock-down of 18 genes associated with the TRAIL pathway

Reqseq nucleotide IDs specified by Aza-blanc *et al* (Aza-Blanc et al. 2003) were used to retrieve mRNA sequences. Sequences were used to search a database of the hairpin sequences contained in the Expression Arrest shRNA library (v1.3) using the BLAST algorithm, via an in house tool for automating BLAST searching (written by Dr. D Beare). Plasmid DNA from each clone was prepared using the Millipore Montage 96 miniprep kit (Millipore #LSKP 096 04) as per manufacturer's instructions. 40ng of pSM2 plasmid DNA for each clone and 40ng pIRES-P was transfected into cells at a density of 12,000 cells per well in triplicate and the sensitivity of cells to TRAIL assayed as described in 2.5.1.2 except media was removed 24 hours after transfection and replaced with HeLa growth media containing 2µg/ml puromycin. Cells were incubated at 37°C, 5% CO₂ for a further 48 hours

before sensitivity to TRAIL was assessed. .

2-4 siRNAs targeting each transcript were ordered from a variety of suppliers. Details of both sequence and source of siRNAs can be found in Appendix A. siRNAs were tested in batches on separate plates with each plate containing siCasp8 and siNeg positive and negative controls in triplicate as described in 2.5.1.2. The siRNAs for each transcript which had the largest effect on TRAIL-induced cytotoxicity were retested on a single plate in triplicate with siCasp8 and siNeg controls as described in 2.5.1.2. .

Statistics were performed by log transforming survival values and performing a one tailed, heteroscedastic t-test between values for each siRNA/shRNA and the siNeg negative control. P-values were adjusted by multiplying by the total number of siRNAs/shRNAs tested.

2.5.7 qRT-PCR of positive control knock-downs

shRNA encoding plasmids and siRNAs targeting TRAIL pathway genes were selected and prepared as described in 2.5.6. Oligonucleotide primers were designed and tested and efficiencies calculated as described in 2.3.7. Primers with more than one product, primer dimers or efficiency below 80% or above 110% were re-designed and re-tested up to three times. Knock-down by each siRNA/shRNA encoding plasmid was measured as described in 2.3.8.

2.6 Chapter 4 and 5 methods

2.6.1 siRNA library screens

For siRNA screening, HeLa cells were grown according to the scheme laid out in Figure 2.1, allowing for cells passaged four times to be available on consecutive days. On the first day cells were seeded for the required number of plates, usually 24 (2.2.7.1). On the second day Library siRNAs were transfected into the plates seeded the day before (2.2.7.2). On the third day media was removed from the first set of plates and replaced with fresh HeLa growth media. On day four the first set of plates were assayed for the viability of cells (2.2.7.4). Plates where siKIF11 did not significantly reduce the viability of cells were discarded. Media was removed and replaced with TRAIL treatment media. On the fifth day viability was reassessed. Sets of plates were overlapped such that day one for the second set of plates was day two for the first set of plates and day one for the third set of plates was day two for the second etc.

For the kinase and phosphatase screen plates 78a through 89b of the Qiagen Druggable Genome siRNA library v2 were transfected into cells in batches of six (replicate 1), 12 (replicate 2) or 24 (replicate 3).

For the Druggable genome screen plates 1a through 77b of the Qiagen Druggable genome library were transfected into HeLa cells in batches of 24. Data was processed (2.4.2) and dynamic ranges for plates calculated. Plates with a dynamic range of less than 2 were repeated.

2.6.2 Reconfirmation of siRNAs from Kinase and Phosphatase screen

siRNAs for confirmation experiments were ordered as pre-arrayed FlexiPlate siRNAs (Qiagen). siRNAs were resuspended in RNase Free Water (Qiagen, included with siRNAs) to a final concentration of 1.25 μ M. siRNAs targeting the first eight genes, plus siCasp8, siNeg and siKIF11 were transfected in quadruplicate on one plate and siRNAs targeting the remaining genes (plus siCasp8, siNeg and siKIF11) were transfected in a second 96 well plate and assayed for sensitivity of 0.5 μ g TRAIL as described in 2.5.1.2.

Statistics were calculated by log transforming survival values for each well and performing a one-tailed, heteroscedastic student's t-test for each siRNA compared with the values for siNeg on the same plate.

2.6.3 qRT-PCR of screen hit siRNAs

Primers were designed to amplify from selected hit transcripts as described in 2.4.1. Primers were tested for specificity and efficiency as described in 2.3.7. Primers that amplified a single band and had an efficiency of between 80% and 110% were used for quantification. Failed primers were not redesigned. Knock-down mediated by hit siRNAs was calculated as described in 2.3.8, except that since measurement of GAPDH and ACTB was split across several qPCR plates, three wells containing reactions with HeLa RNA as a template and either ACTB or GAPDH were included as inter-run controls to allow normalisation of plate differences. .

2.6.4 Luminescent Caspase assays

Protocol described below describes that used for measurement of effect of confirmed hits from kinase and phosphatase screen and effect of over-expression screen hits on Caspases 8,9,3 and 7 and protocol used for measurement of confirmed hits from Druggable gene screen on TRAIL dependent Caspase 8 and Caspase 9 activation. For measurement of effect

of knock-down of hits from druggable genome screen, only three wells per siRNA were transfected and all were treated with TRAIL in step 10.

1. Passage 3 cells were passaged as described in 2.2.1 and resuspended in HeLa Seed media.
2. Cells were diluted to 3×10^4 cellsml⁻¹ in HeLa Seed media.
3. 100µl of cells was added to wells of a 96 well OptiLux white walled tissue culture plate (BD Bioscience #353947).
4. Plates left on bench to allow cells to adhere to plate for 20 minutes.
5. Plates were placed in a semi-sealed polystyrene box stood on petri-dishes of sterile water.
6. Cells were incubated at 37°C, 5% CO₂ for 24 hours.
7. Cells were transfected with 2.5pmol of siRNA as described in 2.2.3 or 80ng plasmid DNA as described in 2.2.4. Each construct was transfected in to six wells.
8. Cells were incubated at 37°C, 5% CO₂ for 24 hours.
9. Media was removed and replaced with 100µl HeLa growth media and incubated for at 37°C, 5% for a further 24 hours.
10. Media was removed. TRAIL treatment media containing 0.5µg/ml TRAIL was added to three of the six wells for each siRNA. TRAIL treatment media containing no TRAIL was added to the remaining three.
11. Cells were incubated for 6 hours at 37°C, 5% CO₂.
12. Caspase-Glo 8 Substrate (Promega # G8200), Caspase-Glo 9 Substrate (Promega #G8210) or Caspase-Glo 3/7 Substrate (Promega, #G8090) was resuspended in Caspase-Glo 8, Caspase-Glo 9 or Caspase-Glo 3/7 buffer (all Promega, catalogue numbers as before).
13. (Caspase-Glo 8 and Caspase 9-Glo only) 7.5µl MG-132 Protease Inhibitor added to substrate.
14. 100µl assay reagent added to each well of plate.
15. Plate sealed and mixed on plate-mixer at lowest speed setting for 2 minutes.
16. Plates incubated for 1 hour.
17. Tape seal removed and luminescence read on Berthold LB96V luminometer with a 10 second integration time.

2.6.5 Measurement of sensitivity of cells to inducers of apoptosis

1. Passage 3 cells were passaged (see 2.2.1) and resuspended in HeLa Seed Media.

2. Cells were diluted to 3×10^4 cellsml⁻¹ in HeLa Seed Media.
3. 100µl of cells were seeded into a 96 well OptiLux white walled tissue culture plate (BD Bioscience #353947). Two wells were left empty for blanking measurements.
4. Plates were placed in a semi-sealed polystyrene box stood on petri-dishes of sterile water.
5. Cells were allowed to adhere to plate for 15 minutes at room temperature.
6. Cells were incubated at 37°C, 5% CO₂ for 24 hours.
7. Cells were transfected with 2.5pmol of siRNA or 80ng plasmid DNA (see 2.2.3 and 2.2.4 respectively). Each siRNA or DNA was transfected into an entire row of cells.
8. Cells were incubated for 24 hours at 37°C for 24 hours.
9. Media was removed by aspiration with a multi-channel aspirator and replaced with 100µl HeLa growth media.
10. Cells were incubated for 24 hours at 37°C.
11. Media was aspirated from cells and replaced with 100µl of alamarBlue assay media.
12. Plates were read in a Gemini SpectraMAX plate reader (Molecular Devices #XPS) using the following settings:
 - Temperature: 37°C.
 - Excitation Wavelength: 544nm.
 - Emission Wavelength: 590nm.
 - Accuracy: 10 readings.

2.6.5.1 TRAIL, FAS and H₂O₂

13. Treatment media was prepared.
 - a. TRAIL media was prepared by diluting 5µg TRAIL in 5ml treatment media per plate to give 1µg/ml TRAIL and serially diluting to give required concentrations.
 - b. FAS media was prepared by diluting 500ng FAS-ligand PlusTM (Calbiochem, # PF092) in 5ml treatment media per plate to give 100ng/ml FAS and serially diluting to give required concentrations.
 - c. H₂O₂ media was prepared by adding 1.13µl 0.3% H₂O₂ solution to 5ml treatment media to give 200nM H₂O₂ and serially diluted to give required concentrations.
14. Media was removed from plate and treatment media added.
15. Plates were incubated at 37°C, 5% CO₂ for 24 hours.

16. Steps 11 and 12 were repeated.
17. Average value of fluorescence in blank wells was subtracted from the fluorescence values in all other wells.
18. Survival of cells in each well was calculated: $survival = \frac{fl_{after}}{fl_{before}} \times 100\%$ where fl_{after} and fl_{before} are the fluorescence readings for that well after and before treatment respectively. .

2.6.5.2 UV

13. Media was aspirated and replaced with 30µl warm PBS.
14. Plate was placed in a Stratalinker UV cross-linker (Stratagene #400075) and lid removed. Columns 10-12 were covered with card.
15. Cells were exposed to 50 Jm⁻² UV.
16. Card was moved to cover columns 7-12 and cells were exposed to 50 Jm⁻² UV.
17. Card was moved to cover columns 4-12 and cells were exposed to 100 Jm⁻² UV.
18. PBS was removed by aspiration and replaced with treatment media.
19. Plates were incubated for 24 hours at 37°C, 5% CO₂.
20. Steps 10 and 11 were repeated.
19. Average value of fluorescence in blank wells was subtracted from the fluorescence values in all other wells.
21. Survival of cells in each well was calculated: $survival = \frac{fl_{after}}{fl_{before}} \times 100\%$ where fl_{after} and fl_{before} are the fluorescence readings for that well after and before treatment respectively.

2.6.6 Re-screen of candidate hits from the druggable genome screen.

Aliquotes of the 40 siRNAs targeting the genes targeted by the top 20 scoring siRNAs were taken from the original library plates. These siRNAs were transfected in triplicate into HeLa cells on several 96 well plates and tested for their sensitivity to 0.5µg/ml TRAIL as described in 2.5.1.2. Each plate also contained three wells transfected with siCasp8, siNeg and siKIF11 respectively.

Statistics were calculated by log-transforming data and then performing a one-tailed, heteroscedastic student's t-test and adjusting the resulting p values by applying Hommel's correction for multiple testing using the `p.adjust()` function in R. .

2.7 Chapter 6 methods

2.7.1 Chromosome 22 ORF screen

The library was provided in 6 plates containing plasmid DNA prepared using Qiagen QIAspin minipreps and diluted to 50µg/ml. Plates were processed in batches of three plates. HeLa cells, passaged 4 times after removal from liquid nitrogen storage, were seeded into assay plates as described in 2.2.7.1. Cells were transfected with 80ng plasmid DNA as described in 2.2.7.3. 48 hours after transfected cells were assayed for viability using the alamarBlue assay (2.2.7.4). Media was removed and replaced with treatment media containing 0.5µg/ml TRAIL. After 24 hours the cells were assayed for viability using the alamarBlue assay (2.2.7.4). Each plate was transfected in two separate replicates.

2.7.2 Creation of the pcDNA3.GW.NoTag vector

The starting point for creating this vector was pcDNA3.GW.V5N adapted from the pcDNA3 (Invitrogen #V790-20) vector by Dr. J. Collins. Since no unique restriction sites exist that will remove tag without also removing the Gateway Cassette, the Gateway cassette was removed first by EcoRV digestion and religation. The V5N tag could then be removed using HindIII and EcoRV double digestion and the Gateway cassette re-inserted. .

1. 2.5µg of pcDNA3.GW.V5N vector digested with 2µl EcoRV in 100µl Buffer 2 for 2 hours at 37°C .
2. Vector religated for 5 minutes at room temperature using the following ligation mix:

<i>Reagent</i>	<i>1X</i>
Digested vector DNA	1µl
5x Roche Dilution buffer*	2µl
Double distilled water	7µl
2x Roche Ligation buffer*	10µl
Roche T4 ligase*	1µl

* From Roche rapid ligation kit (Roche #11 635 379 001).

3. Ligation product was transformed into ccdB sensitive MACH1 chemically competent cells (Invitrogen #C8620-03) and the transformants plated on gentomycin/LB agar plates.
4. Plates were incubated at 37°C for 16 hours.
5. 4 colonies were picked and cultured in 1ml LB/Gentomycin for 8 hours.

6. 6µl of each starter culture was added to 3mls of LB/Gentomycin and cultured at 37°C for 16 hours.
7. Plasmid DNA was prepared from cultures using Qiagen QIAprep spin mini kit (Qiagen #27104) according to manufacturer's instructions.
8. 5µg of plasmid was digested with 5µl HindIII and 5µl EcoRV in 100µl 1x NEB Buffer 2 for 1 hour at 37°C.
9. Reaction product was cleaned up using Qiagen QIAquick PCR clean-up kit (Qiagen #28104) according to manufacturer's instructions.
10. HindIII sticky ends were blunted using T4 polymerase using the following reaction mix assembled on ice:

<i>Reagent</i>	<i>1X</i>
Vector DNA	30µl
NEB Buffer 2	10µl
BSA	0.5µl
2mM dNTPs	5µl
Double distilled water	52.5µl
T4 polymerase (NEB #M0203S)	2µl

11. Reaction was incubated for 15 minutes at 12°C and stopped by adding 10µl 100mM EDTA and heating to 75°C for 20 minutes.
12. Reaction was product purified using QIAquick PCR cleanup kit according to manufacturer's instructions.
13. Product was dephosphorylated by adding 2.5µl Antarctic Phosphatase Buffer and 1.5µl Antarctic phosphatase and incubating for 30 minutes at 37°C.
14. Gateway cassette was ligated into the vector using the following ligation mix:

<i>Reagent</i>	<i>1X</i>
Vector DNA	1µl
Gateway C.1 cassette*	2µl
5x Dilution Buffer [^]	2µl
Double Distilled Water	6µl
2X Ligation Buffer [^]	10µl
T4 ligase [^]	1µl

* from Gateway vector conversion kit (Invitrogen #11828029).

[^] from Roche Rapid Ligation kit.

15. Reaction was incubated at room temperature for 5 minutes.
16. 0.5µl Ligation product was transformed into ccdB resistant DB3.1 chemically competent cells (Invitrogen #11782-018) and transformants plated on chloramphenicol/LB agar plates.
17. Plates were Incubated at 37°C for 16 hours.
18. Ten were colonies picked and cultured in 3mls 25µg/ml chloramphenicol /LB for 16

hours at 37°C.

19. Plasmid DNA was prepared using QIAspin mini prep kit as per manufacturer's instructions.
20. Orientation of Gateway cassette was checked by digesting 1µl plasmid DNA with 1µl EcoRV and 1µl EcoRI in 10µl 1x NEB EcoRI buffer.
21. Products were run on a 1% maxi-gel. If gateway cassette is in correct orientation digestion results in a 1259bp band, if in the wrong orientation results in a 412bp band. 4 clones had cassette in correct orientation.
22. Absence of V5N tag was confirmed by digesting 1µl of plasmid DNA with 1µl EcoRV and 1µl HindIII in 10µl 1x NEB buffer 2. .
23. Products run on a 1% maxi-gel. Presence of V5N tag would have shown as a 1700bp band. No clones contained V5N tag.

2.7.3 Gateway LR recombination reactions

Gateway LR recombination reactions move an insert from a pENTR entry clone into a pDEST destination clone. pENTR vectors and pDEST vectors contain different antibiotic resistance markers allowing for the selection of one or the other in transformation. Un-recombined pDEST clones carry the gateway cassette which contains the ccdB gene which is toxic to most bacterial cell lines, allowing for selection against un-recombined vectors.

1. pENTR DNA was diluted to 7.4µl with double distilled water.
2. pENTR DNA was linearised with 1µl PvuI enzyme in 10µl 1x NEB buffer 3 for 1 hour.
3. LR reactions set up for each of the reactions plus negative and positive (pENTR-gus*) controls from master-mix:

<i>Reagent</i>	<i>1x</i>	<i>7x</i>
pcDNA3.GW.NoTag	0.36µl	2.55µl
100mM Tris EDTA pH 7.4	6.64µl	46.48µl
LR Clonase II*	1µl	7µl
pENTR clone	2µl	-

* from the LR Clonase II kit (Invitrogen #11791020).

4. Reactions were incubated at 25°C for 24 hours and terminated by adding 1µl proteinase K and incubated for 20 minutes at 37°C.
5. 0.5µl of each reaction was transformed into MACH1 cells and transformants plated on gentomycin/LB plates.
6. Plates was incubated for 16 hours at 37°C.
7. Colonies were picked and cultured in 150mls gentomycin/LB for 16 hours at 37°C.

8. Plasmid DNA prepared using Qiagen HiSpeed Maxi kit (Qiagen #12663) according to manufacturer's instructions.
9. Inserts were end sequenced by the in house sequencing service using the primers:
Forward: AATGTCGTAACAACCTCCGCC
Reverse: GCGATGCAATTTCCTCATTT.
10. Sequences were checked against expected ORF end sequences using the cross_match algorithm.

2.7.4 qPCR of ORF expression

Primers were designed to amplify sequences from the ORFs using the primers 3 software. The efficiency of the primers was determined as described in 2.3.7, except cDNA template was supplemented with 1µl of each pENTR clone before serial dilution. .

Cells were seeded and transfected as described in 2.2.6.2 except that cells were transfected with 400ng of pcDNA3.ORF.T7N, pcDNA3.ORF.T7C or pcDNA3.ORF.NoTag for each ORF except for MTMR3 where pcDNA3.ORF.T7N was unavailable, or pcDNA3.T7 as a negative control. RNA was prepared from each sample as described in 2.2.5. RNA isolated from each sample was divided in two. One half was used in a reverse transcription reaction (2.3.6,RT+ sample) while the other half was used in a mock reverse transcription reaction that contained no reverse transcriptase enzyme (RT- sample). The expression of the ORFs in the RT+ sample was determined as described in 2.3.8. In addition 4µl of each RT- sample was used as a template for qPCR using primers for the gene of interest in triplicate (2.3.5). Expression levels were calculated using qBase (Hellemans et al. 2007). Repeats whose Ct value differed more than 0.5 from the other two repeats were excluded. Expression levels were standardised using ACTB and GAPDH levels in RT+ sample, and results were normalized to levels in pcDNA3.T7 sample.

3 A TRAIL APOPTOSIS ASSAY AND COMPARISON OF RNAi SCREENING METHODS

There are many ways in which an RNAi screen can be conducted. RNAi can be induced using chemically synthesised RNA oligonucleotides (siRNAs) or using RNA hairpins (shRNAs) expressed from plasmid vectors. Screens can also be performed using a gene-by-

gene strategy, where the screen is performed in a microtitre plate with each well containing a construct or constructs targeting one gene, or using a pooled strategy, where constructs targeting many genes are introduced into a single population and some form of selection applied, followed by the identification of hairpins that are enriched, after this selection.

This chapter presents firstly, the establishment of an assay for a process of biological and medical interest that can be altered using RNAi, namely an assay for TRAIL-induced apoptosis (see section 1.3). This assay is optimised to provide the greatest differentiation between negative and positive controls for genes whose knock-down alters the function of the pathway.

Secondly, this assay is used to compare two different methods for inducing RNAi – namely transfection of siRNAs and transfection of vectors encoding shRNAs.

3.1 Introduction

3.1.1 Assays for apoptosis

In order to screen for genes involved in TRAIL mediated apoptosis, a method for measuring the apoptosis caused by treatment with TRAIL is required. There are many methods for the detection of apoptosis. These can be divided into two strategies. The first is to utilise methods that measure events that are specifically associated with apoptotic cell death (reviewed in (Huerta et al. 2007)). These include: the examination of cell morphology by electron or fluorescent microscopy, methods for the detection of DNA fragmentation, Annexin V staining, measurement of the activity of apoptosis effector proteins, such as caspases, and the detection of the cleavage targets of caspases. While these methods provide an accurate and reliable readout of apoptosis, they are often time consuming and therefore unsuitable to high-throughput screening applications. Those that can be adopted for a high-throughput screen are generally prohibitively expensive for the academic laboratory.

The second strategy is to measure the loss in cell viability caused by treatment with a known apoptosis inducing agent. While this does not specifically measure the level of apoptosis, interesting results can be followed up using a lower throughput method that specifically measures apoptosis to confirm that this is the cause of the loss of cell viability.

There are several methods for measuring cell viability. The simplest are dye exclusion assays. In these assays cells are stained with dyes such as Trypan Blue or Propidium Iodide, which are excluded from living cells, but readily stain cells with compromised membrane integrity. The number of stained and unstained cells can be counted using microscopy. While

simple, these assays are also time consuming due to the necessity for microscopy. There are several dyes that measure viability by measuring metabolic activity of cells. One such dye is alamarBlue. In its native, oxidised form alamarBlue is a blue, non-fluorescent compound. When reduced by the action of respiring cells on the culture media, the dye becomes red and fluorescent, as measured by excitation at 360-530 nm and monitoring emission at 590nm. alamarBlue requires no processing of samples, is non-toxic to both the cells being studied and the user, and is cheap. An alamarBlue assay for the effect of TRAIL on the viability of HeLa cells was successfully used by Aza-blanc *et al* in a previous screen for regulators of the TRAIL-induced apoptosis pathway (Aza-Blanc et al. 2003). The assay involved transfecting cells in duplicate with the RNAi inducing agent, and then treating one duplicate with the TRAIL ligand and the other with media alone. After 24 hours the viability of the cells in both replicates is measured by incubating the cells for 4 hours in media with 10% alamarBlue and measuring the fluorescent emission from the wells (Figure 3.1a). The effect of the TRAIL on the cells is expressed as the percentage of cells that survive using the following equation:

$$survival = \frac{\bar{f}l_{treated}}{\bar{f}l_{untreated}} \times 100\%$$

Equation 1

where f_l_x is the fluorescent signal of well type x and $\bar{f}l_x$ is the mean of all wells of type x. Where a particular experiment contains several treated and several untreated wells, the average survival is the ratio of two means – the ratio of the mean fluorescence in treated wells to the mean fluorescence in untreated wells. Since the average survival is a ratio of two means the standard deviation of this value cannot be directly calculated, but is estimated thus:

$$\sigma_{survival} = survival \sqrt{\left(\frac{\sigma_{f_l_{untreated}}}{\bar{f}l_{untreated}}\right)^2 + \left(\frac{\sigma_{f_l_{treated}}}{\bar{f}l_{treated}}\right)^2}$$

Equation 2

Where $\sigma_{f_l_x}$ is the standard deviation of the fluorescent signal from wells of type x. This makes the application of statistical methods such as Student t's tests more complex. This only applies in situations where an individual experiment contains multiple replicate wells. For example this does not apply if an experiment is repeated several times, but each individual experiment contains only one treated and one untreated well, particularly if they are both on the same plate. Here the survival in each experiment is simply the ratio of the individual values in each replicate and the mean survival is the mean of the survival values

for each replicate.

Here the survival is a measurement of the change in cell viability rather than a direct measurement of the induction of apoptosis. As such it strictly measures TRAIL-induced cytotoxicity rather than TRAIL-induced apoptosis and the ability to specifically induce apoptosis of any new gene isolated using this assay must be tested.

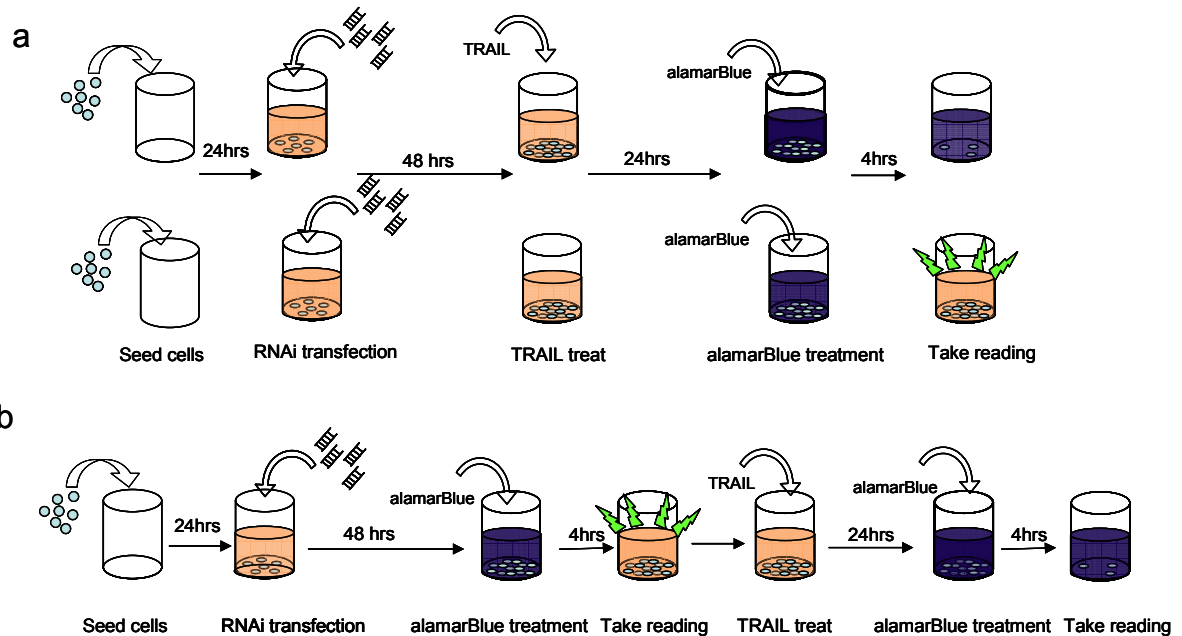


Figure 3.1 TRAIL cytotoxicity assays.

a) Treated vs. untreated assay as used by (Aza-Blanc et al. 2003). Schematic representation of assay. Cells are seeded and transfected in duplicate. After 48 hours one of the two duplicates is treated with TRAIL ligand for 24 hours. The viability of both duplicates is then assessed using alamarBlue. See Equation 1 for calculation of average survival and Equation 2 for estimation of standard deviation. b) Before vs after protocol. Schematic representation of assay. Cells are seeded and transfected. After 48 hours viability is assessed using alamarBlue, followed by treatment with the ligand. Viability is assessed again after 24 hours. See Equation 6 for calculation of survival

3.1.2 The pSHAG-MAGIC vector and the Expression Arrest library

Hairpin encoding vectors in this chapter come from, or are constructed using the same principles as the Expression Arrest Library (v1.3)(Silva et al. 2005) which was kindly made available by Prof. G. Hannon. Constructs in this library are designed to mimic the structure of natural miRNA precursors and are termed shRNA^{mir}s. The constructs were designed by remodelling the human miRNA miR-30 to include a sequence targeting the gene of interest. This remodelled miRNA is inserted into a vector containing 125bp of 5' and 3' sequence from the primary miR-30 transcript. Thus, when transcribed the sense and antisense hairpin structures are flanked by miR-30 leader and termination sequences and linked by the miR-30 hairpin loop. It has been reported that hairpins designed thus are up to 12 times more efficient than standard shRNAs (Silva et al. 2005).

The shRNA^{mir} constructs are cloned into the shRNA^{mir} expression vector pSHAG-

MAGIC2 (pSM2). The vector is based on a self-inactivating Mouse Stem Cell Virus (MSCV). The hairpin is cloned between the viral Long Terminal Repeats (LTR). Hairpins are expressed from the U6 promoter. The vector includes sequences for three selectable markers: kanamycin, chloramphenicol and puromycin. The kanamycin selectable marker is outside the viral LTRs, while the chloramphenicol (for selection in bacterial cells) and puromycin (for selection in mammalian cells) markers are between the viral LTRs. The vector also contains a molecular barcode that allows identification of the vector from within pools. Unfortunately, at the time of writing, the sequences of the barcodes are not available.

Release 1.3 of the Expression Arrest library contains 18,882 bacterial clones containing vectors encoding hairpins targeting 13,456 unique predicted or confirmed Refseq transcripts. The library is provided arrayed in 96 well plates of glycerol stocks with each well containing one clone. The sequence of the hairpins was confirmed before the library was released. In addition 960 clones were sequenced when the library was obtained; 88% of clones contained the expected sequence.

3.1.3 Measurements of the effectiveness of a screen

In order to develop a high-quality assay and compare different screen methods it is necessary to have a metric to assess the effectiveness of a screen. The obvious statistics for assessing the quality of a screen are the sensitivity and accuracy (otherwise known as positive predictive value and related to the false positive rate), defined as follows:

$$sensitivity = \frac{TP}{TP + FN} \times 100\%$$

Equation 3

$$accuracy = \frac{TP}{TP + FP}$$

Equation 4

where TP is true positives, FN is false negatives and FP is false positives. While this information tells us the ultimate success of a screen, it relies on knowing where the true “hits” are, and gives little information on the magnitude of the differences between the true positives and true negatives. Two statistical measures that address these flaws are the signal to noise (S/N) and signal to background (S/B) ratios. They measure the size of the difference between the positive and negative controls. However, they incorporate either no information on variation (S/B), or information on the variation of only one of the values (S/N). Zhang *et al* proposed a new measure of separation between two populations (e.g.

positive and negative values) named the Z'-factor, which is defined as the ratio of the “signal band” to the dynamic range (Zhang, Chung & Oldenburg 1999):

$$Z' = 1 - 3 \frac{\sigma_+ + \sigma_-}{|\mu_+ - \mu_-|}$$

Equation 5

where μ_+ , σ_+ , μ_- and σ_- are the mean and standard deviation of the positive and negative controls, respectively. The value of Z' ranges from 1 (the perfect assay) to $-\infty$. Zhang *et al* propose using the following categories to interpret the Z'-factor:

Z'-Factor	Meaning
1	The perfect assay
0.5-1	An excellent assay which could provide quantitative information
0-0.5	A usable assay that may provide limited quantitative information
0	A Yes/No Assay
<0	Screen essentially impossible

Table 3-3 Interpretation of the Z-factor values

Strictly Standardised Mean Difference (SSMD) has been proposed as an alternative to the Z-factor (Zhang 2007). The main advantage of SSMD over Z-factor is that it has a clear probability interpretation. However, this calculation is only trivial when the data considered is normally distributed. Since the data dealt with here are ratios, and therefore not normally distributed, there would seem to be no advantage in using SSMD over the more widely recognised Z'-factor.

3.2 Demonstration of cytotoxicity of TRAIL

In order to demonstrate the sensitivity of HeLa S3 cells to the TRAIL ligand, and to set up an assay that could be used to compare methods for screening RNAi libraries, the assay used in (Aza-Blanc *et al.* 2003) was adapted. When the assay was directly scaled for a 96-well system the cells showed little or no sensitivity to the TRAIL ligand (see Figure 3.2a). A series of optimisation experiments was undertaken to improve the sensitivity of HeLa S3 cells to the TRAIL ligand. Examples of representative results from this process are presented in Figure 3.2. Factors investigated include the type of plate used (standard tissue culture plate vs. Falcon OptiLux™ white walled tissue culture plate, data not shown), concentration of serum in the assay media (Figure 3.2b), length of treatment (Figure 3.2c,d), the protocol used for seeding the cells (data not shown), number of cells seeded into each well (Figure 3.2d) and concentration of the ligand (Figure 3.2d). It was found that using white-walled plates eliminated fluorescent cross-talk between wells in the plate. Cytotoxicity was improved by a

new seeding protocol which allowed cells to adhere to the surface of the plate before the plate was placed in a moist box and placed in the 37°C, 5% CO₂ incubator. Cytotoxicity was also improved using a lower concentration of serum in the assay medium and using a smaller number of cells (Figure 3.2b and Figure 3.2d). Neither the concentration of the ligand nor the length of treatment seemed to have a large effect on the sensitivity of the cells to TRAIL, except at very low cell densities where treating for 48 hours rather than 24 slightly increased the sensitivity (Figure 3.2c,d). Using 2,500 cells and treating with 1.26µg/ml TRAIL for 24 hours in serum-free media led to a survival of only 31%, and treating for 48 hours led to a decrease in survival to only 13% when compared to untreated cells

These figures compare to an average survival of 38.5% reported previously (Aza-Blanc et al. 2003), where a higher density of cells were used, and cells were treated in medium with 1% serum, and demonstrate that HeLa S3 cells are sensitive enough to allow high-throughput screening for genes that reduce sensitivity to TRAIL ligand.

3.3 Rescue of TRAIL induced cytotoxicity

3.3.1 Rescue of TRAIL induced cytotoxicity by siRNA

Caspase 8 is a key gene in the TRAIL-induced apoptosis pathway (see section 1.3.1). In order to demonstrate that TRAIL-induced cytotoxicity can be modified by RNAi, siRNAs targeting either the Caspase-8 gene (siCasp8) or the Luciferase gene (siGL2), as a negative control, were transfected into various numbers of HeLa S3 cells in varying quantities and the cells were then assayed for TRAIL sensitivity. As discussed above, it was found that lower density cells were more sensitive to the TRAIL ligand than higher cell densities. However, protocols for transfection of siRNAs into cells all specify a higher density of cells than those found to be optimal for the highest sensitivity to the TRAIL ligand. As a compromise cell densities of 10,000 and 5,000 cells per well were tested. It was found that cells transfected with siCasp8 became insensitive to TRAIL-induced cytotoxicity, with greater than 80% of cells surviving 24 hour treatment with TRAIL ligand (Figure 3.3). This was irrespective of the quantity of siRNA transfected into the cells, with the exception of cells at a density of 5,000 cells per well transfected with 10pmol of siCasp8 (Figure 3.3). However, given the large error in that measurement, and that cells at a density of 10,000 cells per well transfected with the same amount of siRNA were resistant to TRAIL-induced cytotoxicity, it seems likely that this datum point is an anomaly possibly due to a failure of transfection. In contrast, cells transfected with siGL2 were sensitive to TRAIL-induced cytotoxicity

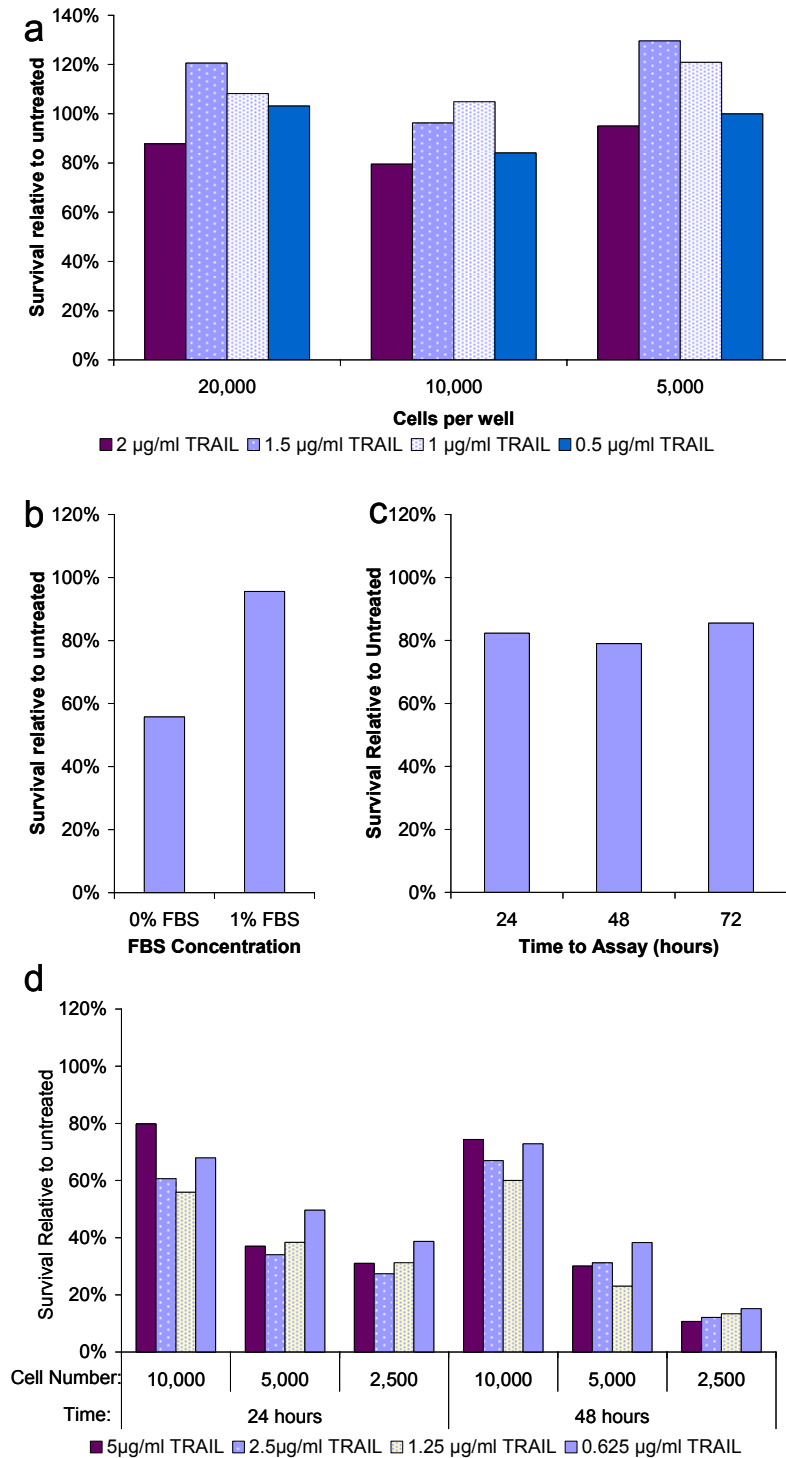


Figure 3.2 HeLa S3 cells are sensitive to TRAIL

a) A direct adaptation of the assay used in by Aza Blanc *et al.* (Aza-Blanc et al. 2003) to a 96 well system. Minimal cytotoxicity was observed under all conditions tried. b) Effect of serum concentration sensitivity to TRAIL. 10,000 cells were seeded and grown for 60 hours and treated with either 1 µg/ml TRAIL or media with concentration of serum indicated for 24 hours and viability assessed using alamarBlue. c) Effect of length of treatment on sensitivity to TRAIL. 10,000 cells were seeded and grown for 60 hours and treated with either 1 µg/ml TRAIL in serum-free media or serum-free media for the length of time indicated and viability assessed using alamarBlue. d) Effect of cell number and TRAIL concentration on sensitivity to TRAIL. Number of cells indicated were seeded and grown for 60 hours and treated with the concentration of TRAIL indicated in serum-free media or serum-free media alone for the length of time indicated and viability was assessed using alamarBlue. All data are means of two replicates.

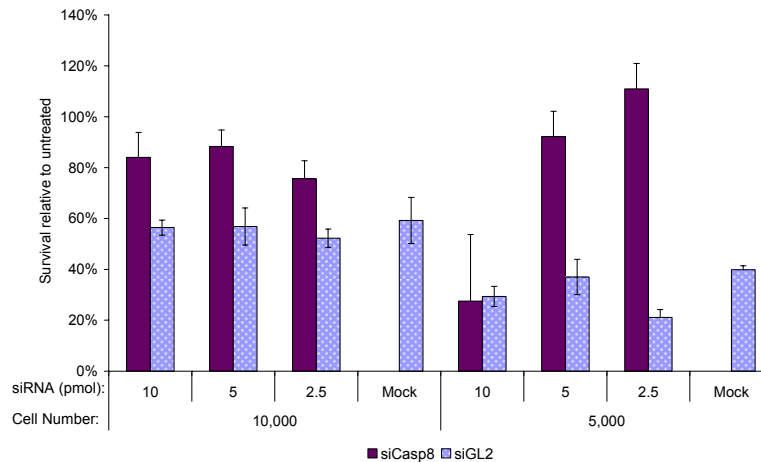


Figure 3.3 siRNA mediated knock-down of Caspase-8 rescues TRAIL-induced cytotoxicity

The number of HeLa S3 cells indicated were seeded and grown for 24 hours. Cells were then transfected with the indicated amount of either siCasp8 or siGL2. After 48 hours cells were treated with either 1 μ g/ml TRAIL in serum-free media or serum-free media alone for 24 hours and viability assessed using alamarBlue. Data are means of three replicates. Error bars represent one standard deviation.

(Figure 3.3). While the density of cells had little effect on the sensitivity of cells transfected with siCasp8, negative control transfected cells at a density of 5,000 cells per well, were more sensitive to TRAIL-induced cytotoxicity than those at a density of 10,000. All cells seeded at a density of 5,000 cells per well and transfected with 2.5pmol of siCasp8 survived 24 hours of treatment with TRAIL, compared to 21% of cells seeded at the same density and transfected with the same quantity of siGL2. These results demonstrate that RNAi targeted against TRAIL pathway members can disable the pathway and rescue cells from TRAIL-induced cytotoxicity.

3.3.2 Construction of pSM2.shCasp8.1/2

In order to test the effectiveness of plasmid based expression of shRNA^{mir}s in modulating TRAIL-induced cytotoxicity, plasmid vectors containing sequences encoding hairpins targeting the Caspase 8 gene were constructed in the vector pSHAG-MAGIC-2c (pSM2). The sequence used to synthesise siCasp8 is a 21bp sequence, whereas the sequences used to generate shRNA^{mir} insets are 22bp sequences and have mismatches at the 3' end of the guide strand. Since simply extending the siCasp8 sequence by a base in the 5' or 3' direction may result in a suboptimal hairpin sequence, three new sequences targeting Caspase 8 were designed using shRNA retriever tool (<http://katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=shRNA>). These sequences were synthesised as 97bp oligonucleotides including miR30 leader, termination and loop sequences. These oligonucleotides were PCR amplified using oligonucleotides containing miR30 leader/termination sequences and either an EcoRI or an XhoI restriction

site. The PCR product was A-tailed and cloned into the holding vector pGEM-T using TA cloning. Several colonies containing the pGEM-hairpin constructs were sequenced to verify the sequence of the hairpin. Hairpins containing the correct sequence were sub-cloned into pSM2 by digesting the pGEM-hairpin construct with EcoRI/XhoI, purifying the fragment of the correct size from an agarose gel and ligating into pSM2 vector digested with the same enzymes. The final constructs were verified by sequencing. In this manner two vectors were successfully constructed which expressed two different hairpins targeting Caspase 8 – pSM2.shCasp8.1 and pSM2.shCasp8.2 (see 2.5.3).

3.3.3 Optimisation of DNA transfection

In order to use plasmid-based shRNA^{mir}-mediated gene silencing to modulate TRAIL-induced apoptosis, it is necessary to introduce the plasmids into the cells being studied at a high efficiency. To test the efficacy of a range of lipid-based transfection reagents, HeLa S3 cells were transfected with plasmid expressing an enhanced GFP protein (pEGFP), allowing for the visualisation of transfected cells. The reagents Lipofectamine 2000 and Effectene gave appreciable transfection efficiency of 36% and 38%, respectively, as calculated by the ratio of the number of green fluorescent cells (number of cells transfected) to the number of DAPI-stained nuclei (total cell number). However, these reagents were associated with very high toxicities, as calculated by the ratio of DAPI-stained nuclei in wells transfected with pEGFP to the number of DAPI-stained nuclei in untransfected wells. In total this gave a very small total number of transfected cells. In contrast, cells transfected with either GeneJuice or siPort XP-1 showed much lower toxicities, but little or no transfection, again resulting in a very small total number of transfected cells (**Table 3-4**).

	<i>HeLa S3</i>		<i>HeLa</i>	
	<i>Transfection Efficiency</i>	<i>Viability</i>	<i>Transfection Efficiency</i>	<i>Viability</i>
Lipofectamine 2000	36.80%	2.90%	37.50%	3.40%
Effectene	48.50%	7.90%	12.86%	3.40%
GeneJuice	0.50%	43.10%	8.90%	55%
siPort XP-1	1.34%	56%	12.20%	61.20%

Table 3-4 Comparison of transfection reagents.

HeLa S3 cells or HeLa cells were seeded on an 8 well slide and grown for 24 hours. Cells were then transfected with pEGFP-N1 plasmid using the indicated transfection reagent. Cells were fixed and stained with DAPI after 48 hours. Transfection efficiency was calculated as the ratio of fluorescence cells to DAPI-stained nuclei. Viability was calculated as the ratio of DAPI-stained nuclei in transfected wells to DAPI-stained nuclei in untransfected wells.

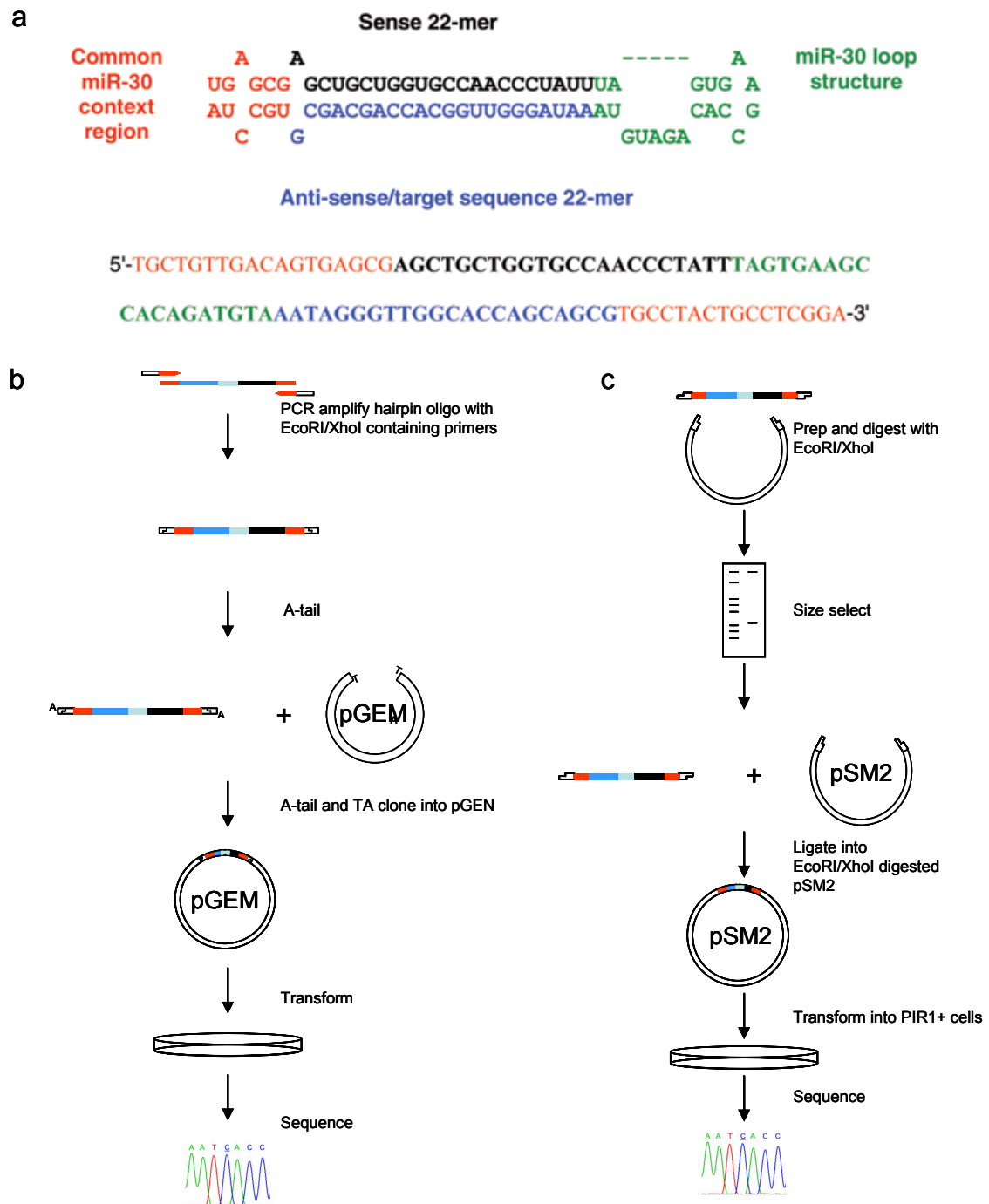


Figure 3.4 Cloning of shRNA^{mir} constructs in the pSM2 vector

a) Above, the structure of the hairpin RNA. Below, the structure of the DNA oligonucleotide template used to generate the constructs. Colours represent the origin of the sequence: Red - miR-30 flanking sequence. Green – miR-30 loop sequence. Blue: sense and anti-sense target sequence respectively. Taken from (Paddison et al. 2004a). b) Cloning of hairpin insert into pGEM holding vector, see text and methods for details. c) Subcloning of hairpin insert into pSM2 by restriction digest. See text and methods for details.

In order to determine if better transfection efficiency could be achieved, the experiment was repeated using HeLa cells. HeLa cells are the parent cell line of HeLa S3 cells and are larger, flatter and adhere to the growth surface more tightly. Transfections using Lipofectamine 2000 and Effectene gave similar results in HeLa cells to those obtained in

HeLa S3 cells. However, transfection using both GeneJuice and siPort XP-1 gave transfection efficiencies an order of magnitude greater than those observed in HeLa S3 cells, while still showing the same low toxicity (**Table 3-4**). To determine if HeLa cells could be used in the TRAIL assay instead of HeLa S3 cells, the sensitivity of HeLa cells was assessed. At higher cell densities HeLa cells seemed insensitive to TRAIL-induced cytotoxicity. However, at very low densities similar numbers of cells survived TRAIL treatment to those seen for 5,000 HeLa S3 cells. Again, both concentration of TRAIL and length of treatment had minimal effect on the sensitivity of the cells (Figure 3.6)

The pSM2 plasmid contains a puromycin resistance marker. Reasoning that selecting for transfected cells after transfection might increase the transfection efficiency by reducing the number of untransfected cells, cells were co-transfected with pEGFP-N1 and pSM2 and then treated with either puromycin for 24 hours to select transfected cells, or grown in media alone for the same length of time.

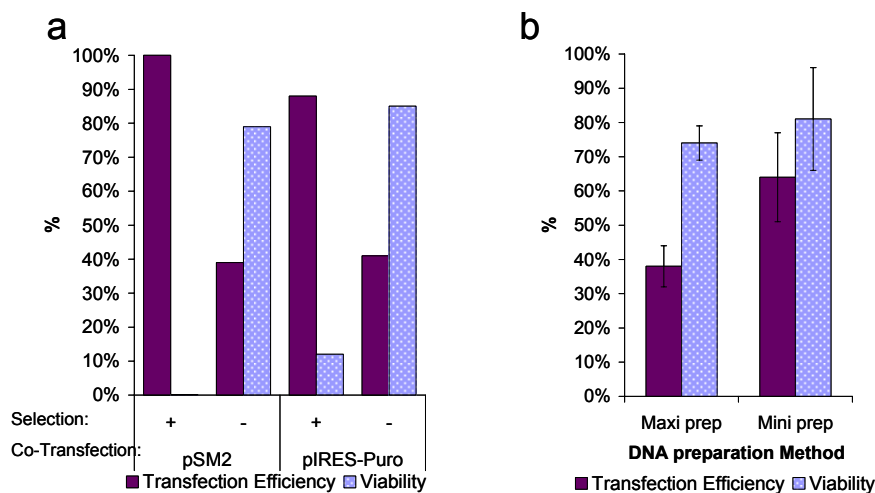


Figure 3.5 Selection of transfected cells using puromycin.

a) Effect of drug selection on transfection efficiency. HeLa cells were seeded on 8 well slides and grown for 24 hours. Cells were co-transfected with either pEGFP-N1 and pSM2 or pEGFP-N1 and pIRES-P. After 24 hours cells were treated with 2 μ g/ml puromycin. Slides were fixed and stained after a further 24 hours. b) Effect of DNA preparation method on transfection efficiency. HeLa cells were seeded on 8 well slides and grown for 24 hours. Cells were then transfected with pEGFP-N1 prepared using Qiagen Plasmid Mini kit or Qiagen Endotoxin-free HiSpeed Maxi kit. Slides were fixed and stained with DAPI 48 hours later. Data are means of three replicates; error bars represent one standard deviation. Transfection efficiency was calculated as the ratio of fluorescence cells to DAPI-stained nuclei. Viability was calculated as the ratio of DAPI-stained nuclei in transfected wells to DAPI-stained nuclei in untransfected wells.

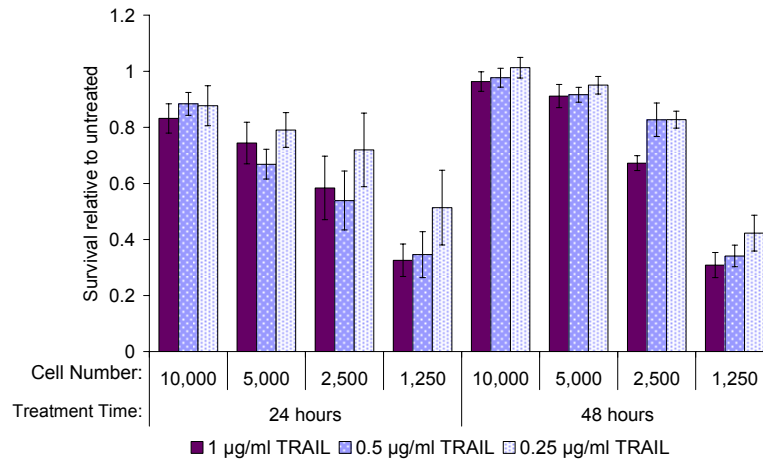


Figure 3.6 HeLa cells are sensitive to TRAIL.

The numbers of HeLa cells indicated were grown for 72 hours. Cells were then treated with either the concentration of TRAIL as indicated, in serum-free media or serum-free media alone for the length of time indicated and viability was assessed using alamarBlue. Data are means of three replicates. Error bars represent 1 standard deviation

Where cells were not selected, transfection efficiencies of approximately 40% were observed. Selection for pSM2 transfected cells using puromycin gave near zero surviving cells. In contrast, 12% of cells co-transfected with pEGFP-N1 and pIRES-p (another plasmid encoding a puromycin resistance marker) were resistant to puromycin selection. Furthermore, these surviving cells were significantly enriched in transfected cells (88% for selected cells compared with 41% for unselected cells, **Figure 3.5c**). These results demonstrate that selecting puromycin resistant cells can increase transfection efficiency when a vector with functional puromycin resistance was used. However, it seems that for unexplained reasons this is not the case for pSM2. One difference between the pIRES-P plasmid and the pSM2 plasmid was the method used to prepare the DNA. The pIRES-p plasmid was prepared using an endotoxin-free HiSpeed maxi prep kit from Qiagen, while pSM2 was prepared using a plasmid mini prep kit from Qiagen. To investigate if this could be the source of the differences seen, pEGFP-N1 plasmid DNA prepared with either an endotoxin-free HiSpeed maxi prep kit or a plasmid mini prep kit was transfected in to HeLa cells and the transfection efficiency assessed. The method of DNA preparation had no significant effect on the efficiency of the transfection ($p = 0.41$, **Figure 3.5d**).

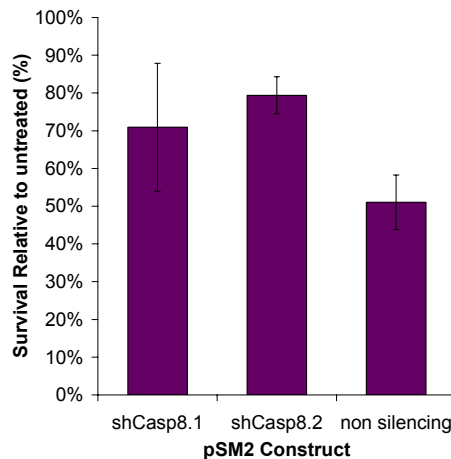


Figure 3.7 shRNAmir mediated knock-down of Caspase-8 rescues TRAIL-induced cytotoxicity

2,500 HeLa cells were seeded and grown for 24 hours. Cells were then transfected with pSM2.shCasp8.1, pSM2.shCasp8.2 or pSM2.shControl (a hairpin targeting a pseudo-gene) using siPort XP-1. After 48 hours cells were treated with either 1 μ g/ml TRAIL in serum-free media or serum-free media for 24 hours. Viability was assessed using alamarBlue. Data are mean of three replicates. Error bars represent 1 standard deviation.

In order to demonstrate that TRAIL-induced cytotoxicity can be modulated by shRNA^{mir} mediated RNAi, HeLa cells were transfected with pSM2 constructs targeting either the Caspase 8 gene (see 0) or a pseudogene (shControl), and the sensitivity of cells to TRAIL ligand was assessed. 51% of cells transfected with the control construct survived 24 hours treatment with TRAIL ligand, while 71% and 79% of cells survived when transfected with shCasp8.1- and shCasp8.2-containing constructs (Figure 3.7) — a 1.4-fold and 1.54-fold relative increase in survival, respectively

The assay used by (Aza-Blanc et al. 2003) and above compares the average viability of treated cells to that of untreated (Figure 3.1a). This method of calculating survival has several drawbacks. Firstly, since the survival is expressed as a ratio of averages, the standard deviation must be estimated from the standard deviations of the average viability of treated and untreated wells. Secondly, all transfections must be carried out in duplicate, using twice the amount of siRNA, transfection reagent, assay reagent etc. Thirdly, while comparing treated to untreated wells takes account of the effect of the siRNA on the basal viability of the cells, it does not take account of variation in the seeding of cells between wells. An alteration of the assay to compare viability before and after treatment in the same well addresses these issues. Here cells are seeded and grown for 24 hours before being transfected. After another 48 hours their viability is assessed and they are treated with TRAIL. The viability is reassessed 24 hours later (Figure 3.1b). Here the survival is calculated per well, using:

$$survival = \frac{f_{after}}{f_{before}} \times 100\%$$

Equation 6

and the average survival is simply the mean of the survival in each well and the standard deviation of survival is the standard deviation of survival in each well.

This new assay protocol was used to study the effect of cell density and the ratio of DNA to transfection reagent on both transfection and assay efficiency simultaneously (Figure 3.2). Increasing the density of cells in both siRNA- and shRNA^{mir}-mediated experiments increased the survival of cells transfected with both control constructs and constructs targeting Caspase 8 or BID, another gene in the TRAIL apoptosis pathway (Figure 3.9a and Figure 3.8b). At 3000 cells per well 38% of cells transfected with pSM2.shControl and 23% of cells transfected with siGL2 survived, while 76% of cells transfected with pSM2.shCasp8.2 and 84% of cells transfected with siCasp8 survived. Transfection of an siRNA targeting BID had a similar but slightly less powerful effect, with 66% of cells surviving, while transfection with pSM2.shCasp8.1 had little effect with 44% of cells surviving.

It is to be noted that a higher percentage of untransfected cells survived than cells transfected with any siRNA or pSM2 construct (Figure 3.8a and Figure 3.8b). However, the same is also true of cells transfected with pEGFP-N1. This suggests that the effect is not due to the engagement of the RNAi machinery, but to the process of transfection itself. It is possible that here toxicity associated with the transfection is affecting the assay (Table 3-4) suggesting where there is no transfection, the cell number is higher than in transfected wells at the time of treatment. However, since these non-specific effects seem to be related to the transfection rather than the construct transfected, the effects should be similar, independent of the siRNA/shRNA^{mir} transfected. Therefore it can be assumed that the differences between different siRNAs/shRNA^{mir}s are the result of the differing effects of that construct on the pathway. This underlines the importance of comparing the effect of an siRNA/shRNA^{mir} to a negative control siRNA/shRNA^{mir} rather than an untransfected sample. The effect of the amount of siRNA transfected on assay outcome has already been investigated. above (Figure 3.3). Using both greater quantities of DNA and greater quantities of transfection reagent increased the difference in TRAIL sensitivity between control transfected cells and cells transfected with pSM2.Casp8.2. A total of 15% of cells transfected with 80ng of pSM2.shControl, using 16 µl of siPort XP-1, survived 24 hours treatment with 1µg/ml TRAIL, while 36% of cells transfected in the same way with pSM2.shCasp8.2

survived. — a 2.4-fold relative increase in survival.

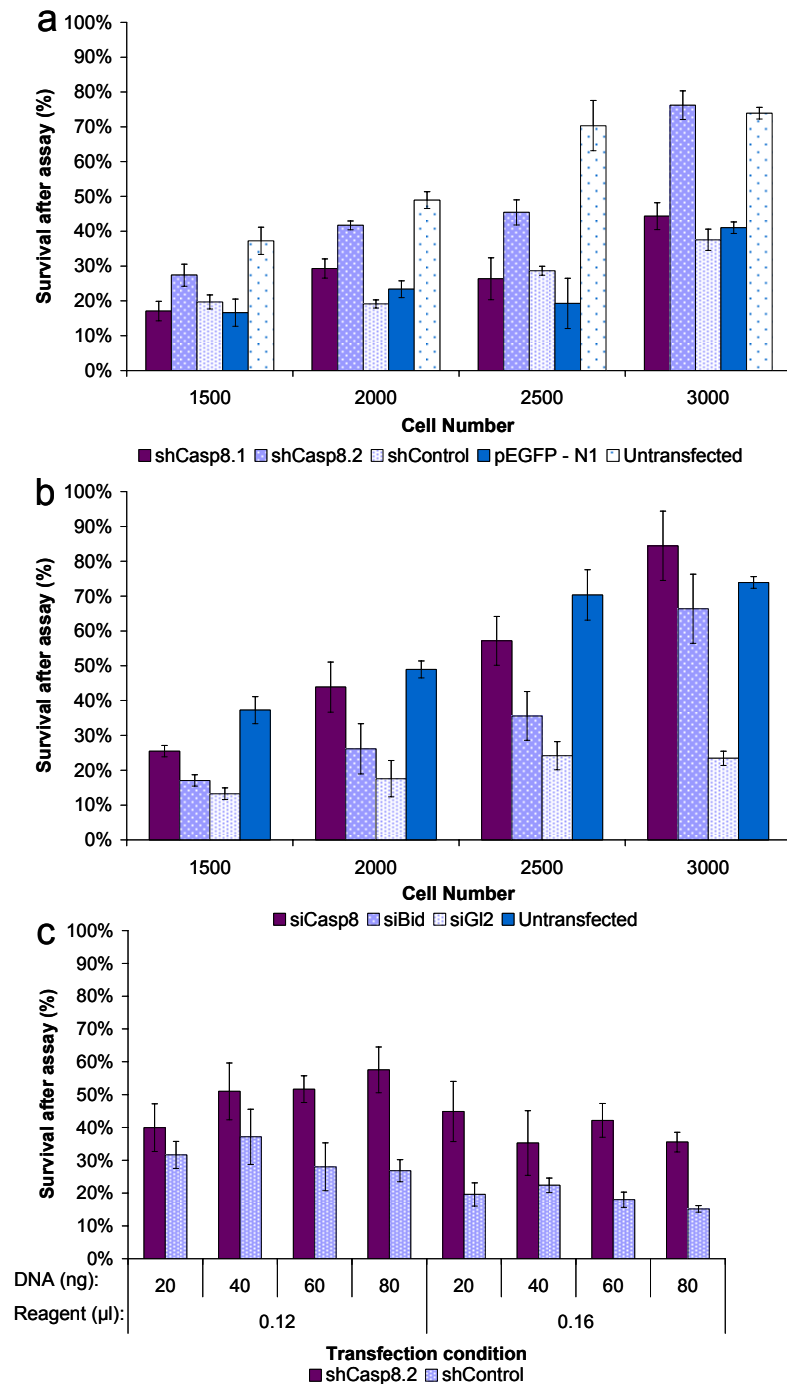


Figure 3.8 Optimisation of RNAi-mediated rescue from TRAIL-induced cytotoxicity .

a),b) Effect of cell number on rescue from TRAIL-induced cytotoxicity. The number of cells indicated were seeded and grown for 24 hours. Cells were transfected with either a) shRNA^{mir} or b) siRNA. After 48 hours cells pre-treatment viability was assessed and cells treated with 1µg/ml TRAIL. Post-treatment viability was assessed 24 hours later. c) Effect of DNA to reagent ratio on shRNA-mediated rescue from TRAIL-induced cytotoxicity. 3000 cells were seeded and grown for 24 hours. Cells were then transfected with the indicated shRNA using amounts of plasmid DNA and transfection reagent as indicated. Cells were grown for a further 48 hours and pre-treatment viability assessed. Cells were then treated with 1µg/ml TRAIL for 24 hours and post-treatment viability was assessed. Error bars represent 1 standard deviation.

Reverse transfection protocols — where cells are overlaid on previously prepared DNA/lipid complexes — have the advantage of saving 24 hours on the traditional transfection process, where DNA/lipid complexes are added to cultures of cells seeded 24 hours earlier. To investigate the feasibility of using this technique here, cells were transfected with pSM2.Casp8.2 or pSM2.shControl under a large range of conditions and the sensitivity to TRAIL was assessed 48 hours later. On average 27% of cells seeded at a density of 4,000 cells per well transfected with 80ng of pSM2.shControl, using 0.24 μ l of siPort XP-1, survived 24 hours treatment with 1 μ g/ml TRAIL, while 56% of cells seeded and transfected in the same way with pSM2.shCasp8.2 survived TRAIL treatment (Figure 3.9) — a 2.07-fold relative increase in survival. This suggests that the reverse transfection protocol is nearly as efficient as the traditional protocol, which require an additional 24 hours.

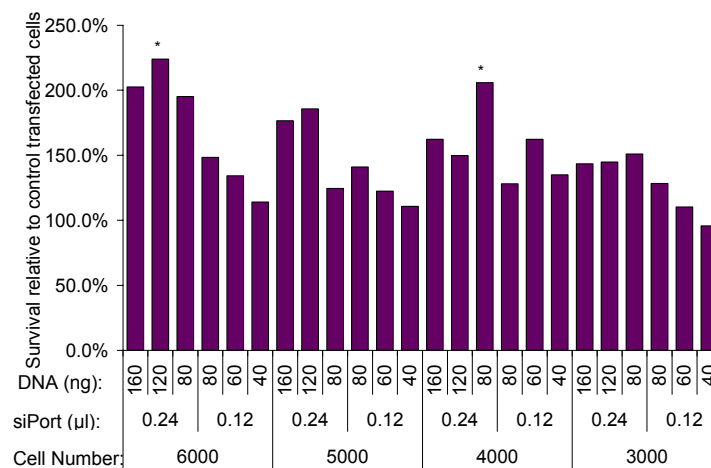


Figure 3.9 Optimisation of RNAi-mediated rescue from TRAIL-induced cytotoxicity using reverse transfection

Transfection complexes were prepared using the amount of DNA and reagent (siPort XP-1) indicated and arrayed in a microtitre plate. The number of cells indicated were seeded on top of the transfection complexes and grown for 48 hours. Pre-treatment viability was assessed and cells were treated with 1 μ g/ml TRAIL. Post-treatment viability was assessed after 24 hours. Results are expressed as the ratio of survival in pSM2.Casp8.2 transfected wells to the survival in pSM2.shControl transfected wells. * marks the conditions giving the greatest difference between pSM2.shCasp8.2 and pSM2.shControl transfected wells. Data are means of two replicates.

Thus, an assay for the assessment of shRNA^{mir}s and siRNAs on the cytotoxicity of TRAIL ligand on HeLa cells has been established and optimised to give the greatest difference between cells with a knock-down of Caspase-8 and cells with a control knock-down.

3.3.4 Confirmation of Caspase-8 mRNA knock-down

In order to confirm that the effect of transfection with shRNA^{mir}s and siRNAs on TRAIL-induced cytotoxicity is due to a knock-down of the Caspase 8 transcript, the levels of the transcript were measured using quantitative reverse-transcription PCR (qRT-PCR).

Transfection with pSM2.Casp8.1, pSM2.Casp8.2 and siCasp8 led to clear reductions in the level of Caspase 8 transcript as compared to levels of transcript in cells transfected with pSM2.shControl (Figure 3.10). The level of knock-down seen correlated with the different size of effects on TRAIL-induced cytotoxicity when cells are transfected with the same constructs. Transfection with siCasp8, which reduced the Caspase 8 transcript to 10% of control had the largest effect on TRAIL-induced cytotoxicity, with siCasp8 transfection increasing the survival of TRAIL treated cells from 23% to 84%. Transfection with pSM2.Casp8.2, which reduced the transcript to 20% of control levels, had a smaller effect on TRAIL-induced cytotoxicity, where transfection increased the survival of cells after treatment with TRAIL from 38% to 76%; whereas, transfection of pSM2.Casp8.1, which only reduced transcript levels to 34% of control, had little or no effect on the survival of cells treated with TRAIL (Figure 3.8a and Figure 3.8b). These results demonstrate that transfection with pSM2.Casp8.1, pSM2.Casp8.2 and siCasp8 does lead to a reduction in Caspase 8 levels and therefore that the effect on TRAIL-induced cytotoxicity is very likely caused by this reduction.

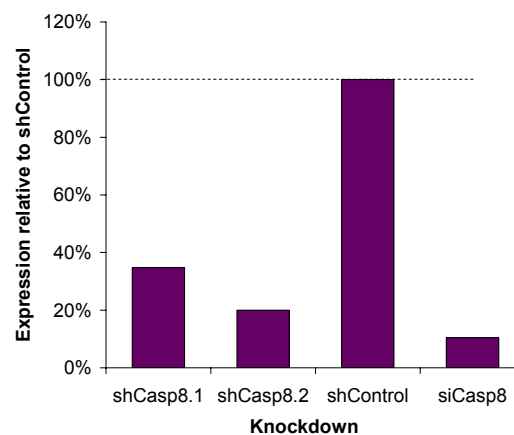


Figure 3.10 qRT-PCR confirmation of Caspase 8 knock-down

RNA was prepared from cells transfected with pSM2.shCasp8.1, pSM2.shCasp8.2, pSM2.shControl or siCasp8. RNA was reverse transcribed to generate cDNA. SYBR green qPCR was carried out on each sample using primers designed to amplify a section of the Caspase 8 transcript or primers designed to amplify a section of the ARSA transcript as control. Amplicons were designed to include large introns in the genomic sequence so that primers would amplify from cDNA but not genomic DNA. Relative levels were calculated using the Pfaffl method (Pfaffl 2001) with ARSA levels used to normalise between samples. Dashed line indicates 100% expression compared to cells transfected with pSM2.shControl.

3.4 Comparison of siRNA and shRNA^{mir} performance

3.4.1 Comparison of the effect of siRNAs and shRNA^{mir}s against Caspase-8 on TRAIL induced cytotoxicity

To compare the effectiveness of shRNA^{mir}s and siRNAs, cells were transfected with either pSM2.shCasp8.2 or siCasp8 and an appropriate non-targeting control, and the effect of treatment with a range of concentrations of TRAIL was tested. Cells were transfected with pSM2.shRNA^{mir} constructs using both a reverse transfection protocol and a traditional transfection protocol. Experiments were carried out in triplicate on one plate, and each experiment was repeated with four separate aliquots of cells, defrosted and grown independently. When cells were transfected with pSM2.Casp8.2 using a reverse transfection protocol, there was a small reduction in TRAIL-induced cytotoxicity (Figure 3.11a) at each concentration tested, with survival of cells after treatment with 1µg/ml TRAIL being increased from 29% for control transfected cells, to 40% for pSM2.Casp8.2 cells — a 38% increase. When cells were transfected using a traditional transfection protocol, there was a larger reduction in cytotoxicity, with a larger proportion of cells transfected with pSM2.Casp8.2 surviving treatment with all concentrations of TRAIL (Figure 3.11b). At 1µg/ml TRAIL, 51% of pSM2.Casp8.2-transfected cells survived, compared to 28% of control-transfected cells — a 92% increase. However, the siCasp8 siRNA performed better than the shRNA^{mir}s under all conditions (Figure 3.11d), with 96% of siCasp8-transfected cells surviving treatment with 1µg/ml TRAIL compared with 29% of control-transfected cells — a 274% increase. One explanation for why the shRNA^{mir}s may perform less well than the siRNA is a difference in transfection efficiency between the shRNA^{mir}s and the siRNAs. Selection of pSM2-transfected cells using the puromycin marker on the vector does not improve the proportion of cells transfected; however, an increase in effective transfection efficiency can be achieved by co-transfecting with the pIRES-P vector, which contains a functional puromycin resistance gene (Figure 3.11c). To increase the transfection efficiency of the pSM2 transfected cells, cells were co-transfected with pIRES-P and either pSM2.shCasp8.2 or pSM2.shControl, and puromycin resistant cells were selected before assessment of TRAIL-induced cytotoxicity. This did not increase the survival of cells transfected with pSM2.shCasp8.2 when treated with TRAIL, with 39% of pSM2.Casp8.2 transfected cell surviving. However, the relative increase in survival after TRAIL treatment between cells transfected with pSM2.shCasp8.2 and those transfected with pSM2.shControl was greatly increased and was similar to the fold difference in survival between cells

transfected with siCasp8 and those transfected with siGL2, with 328% more cells surviving after pSM2.Casp8.2 transfection than in control transfection. This is due to a decrease in the survival of cells transfected with pSM2.shControl (Figure 3.11c), with only 9% of cells surviving treatment with 1 μ g/ml TRAIL.

It can therefore be concluded that siRNAs are more effective than shRNA^{mir}s at altering the function of the TRAIL-induced cytotoxicity pathway. This difference can be reduced in terms of dynamic range, but not in terms of absolute magnitude, by co-transfecting with a puromycin resistance marker and selecting puromycin resistant cells.

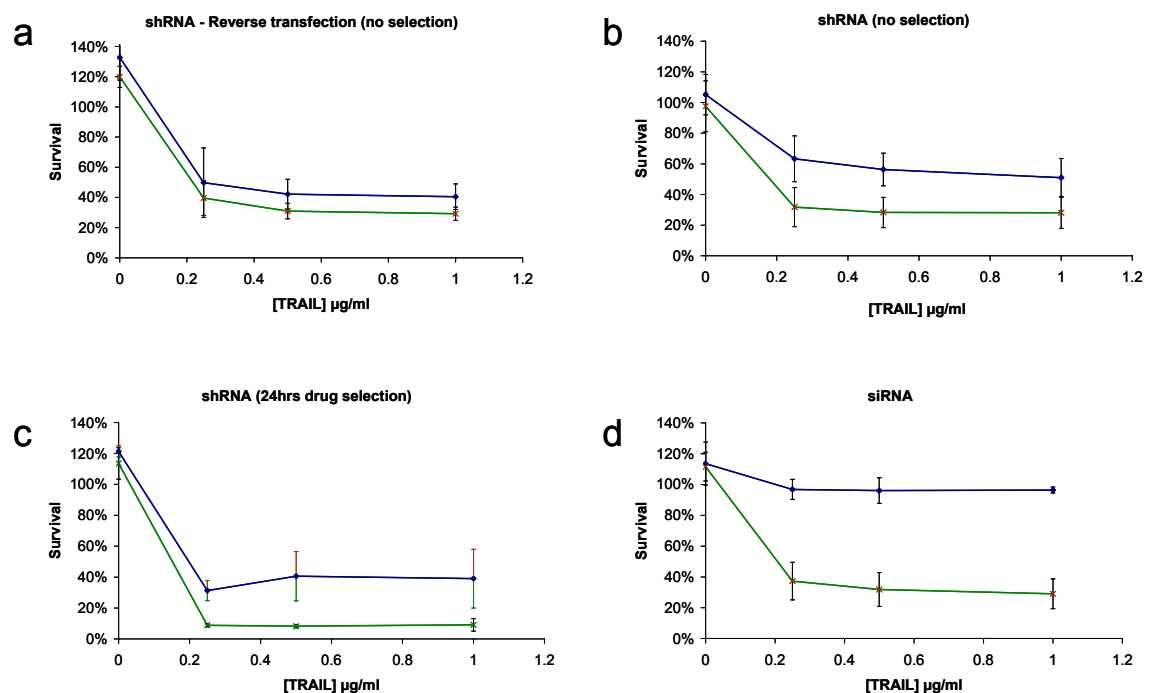


Figure 3.11 Comparison of the effects of shRNA- and siRNA-mediated knock-down of Caspase 8 on TRAIL-induced cytotoxicity.

Cells were transfected with shRNAs or siRNA targeting Caspase 8 (blue line) or a negative control (Green line) and sensitivity to TRAIL was assessed by measuring viability using alamarBlue and treating cells with a range of TRAIL concentrations. Viability was reassessed 24 hours later. a) 4,000 cells were seeded and simultaneously transfected with 80ng of shRNA^{mir} expressing construct using a reverse transfection protocol. TRAIL sensitivity was assessed 48 hours later. b) 3,000 cells were seeded and grown for 24 hours. Cells were transfected with 80ng of shRNA^{mir} constructs. TRAIL sensitivity was assessed 48 hours later. c) 12,000 cells were seeded and grown for 24 hours and transfected with 40ng of shRNA^{mir} and 40ng of pIRES-P. After 24 hours, transfected cells were selected by treatment with 2 μ g/ml puromycin. TRAIL sensitivity was measured 48 hours later. d) 3,000 cells were seeded and grown for 24 hours. Cells were transfected with 2.5pmol siRNA. TRAIL sensitivity was assessed 48 hours later. All points are means of four biological replicates. Error bars represent 1 standard deviation

3.4.2 Blind pseudo-screens show a clone-by-clone screen is practical with siRNA, but not shRNA^{mir}s

Although siRNAs targeting Caspase 8 have a larger effect on cytotoxicity induced by treatment with the TRAIL ligand than shRNA^{mir}s targeting the same gene, this does not imply that the siRNA mediated effect is large enough to allow an effective screen, or that the

shRNA^{mir} mediated effect is not large enough. The feasibility of performing clone-by-clone screens using both shRNA^{mir}s and siRNAs was examined by using a blind pseudo-screen. Here, siRNAs/shRNA^{mir}s were arrayed in a 96-well plate by a colleague in such a way that the plate contained 5-15 wells with siRNAs/shRNA^{mir}s targeting a positive control (Caspase 8 in this case) and all other wells filled with negative controls. Without knowledge of the location or number of the positive controls on the plate, the constructs were transfected into a 96-well plate of cells and the level of cytotoxicity induced by treatment with the TRAIL ligand was assayed. If the effect of the knock-down is large enough to allow screening, it should be possible identify the number and position of wells containing positive controls. As well as measuring the success of this experiment in terms of sensitivity and accuracy, a *Z'* score (see section 3.1.3) can be calculated and used to compare different protocols.

Blind pseudo-screens were carried out using shRNA^{mir}s, shRNA^{mir}s co-transfected with pIRES-p and selection applied for 48 hours with puromycin, or using siRNAs. When shRNA^{mir}s were used 70% of the positive controls were identified, while 60% of the wells selected were false positives. This was improved by using puromycin to select cells co-transfected with pIRES-p, allowing 77% of the positives to be selected without the selection of any false positives. In contrast, 100% of wells transfected with the siRNA positive control were selected without the selection of any false positives. The difference is even greater when *Z'* factors, calculated from the values of all wells on the plate, are considered. The *Z'* factors were -0.06, -0.7 and 0.46, for shRNA^{mir}s without selection, shRNA^{mir}s with selection and siRNAs, respectively (Figure 3.12). *Z'* scores of less than 0 are generally thought to indicate an assay of limited use, while those with a *Z'* prime score of greater than 0.5 are thought to be excellent assays by those involved in chemical screening (Zhang, Chung & Oldenburg 1999).

Thus siRNAs targeting Caspase 8 outperform shRNA^{mir} targeting the same gene by a wide margin when considering *Z'* scores, with the *Z'* score for siRNAs coming close to that thought to indicate an excellent assay (Zhang, Chung & Oldenburg 1999). The consequence is seen in the number of positives picked out in the blind pseudo-screen. From this it can be concluded that screening with siRNAs is feasible and offers the possibility of an accurate and sensitive screen for genes involved in TRAIL-induced cytotoxicity. In contrast, shRNA^{mir}s, whether or not drug selected, do not provide the necessary power to distinguish genes that affect TRAIL-induced cytotoxicity compared to those that have no effect, particularly since Caspase 8 was the gene that had the largest effect on TRAIL-induced cytotoxicity in previous screens (Aza-Blanc et al. 2003).

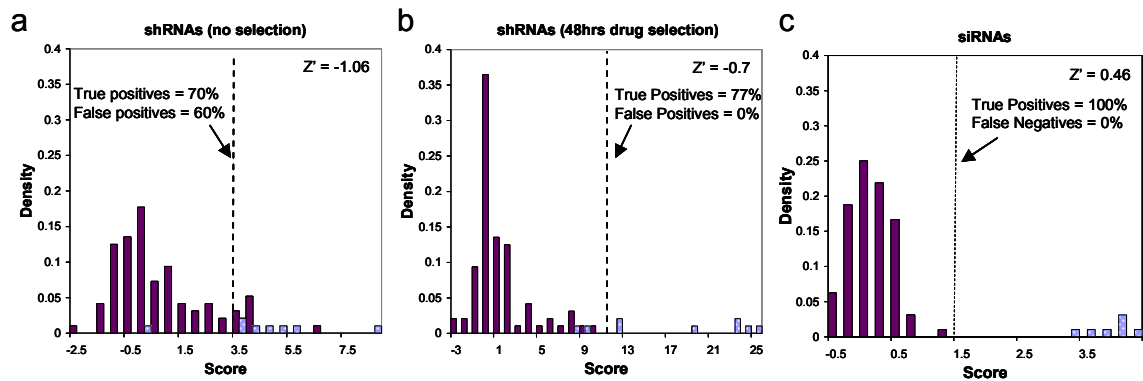


Figure 3.12 Histograms showing distributions of positive and negative controls in blind pseudo-screens. Cells were seeded and grown for 24 hours then transfected with shRNAmir expressing vectors (a), co-transfected with pIRES-P and shRNAmir expressing vectors (b) or siRNAs (c), targeted against either Caspase 8 or a negative control. The identity of the siRNAs/shRNAmirs was unknown to the experimenter (see text). Cells were either grown for 48 hours and assessed for sensitivity to $1\mu\text{g/ml}$ TRAIL (a and c) or grown for 24 hours and then treated with $2\mu\text{g/ml}$ puromycin for 48 hours before being assessed for sensitivity to $1\mu\text{g/ml}$ TRAIL. Standardised scores were calculated for each well using robust estimates for μ and σ . An experimenter selected cut off for hits was selected by manual inspection. (dashed line). After positions of positives were revealed, Z' factors between positive controls (light bars) and negative controls (dark bars) were calculated using robust estimators for μ and σ .

3.4.3 Differences in assay outcome between siRNAs and shRNA^{mir}s are due to different knock-down efficiencies for a wide range of positive controls

Until this point all experiments have examined only the effect of knocking down one gene – Caspase 8. To study the effects of knocking a larger selection of genes, 18 genes that have previously been implicated in TRAIL-induced cytotoxicity (see Table 1-2) and were present in the Expression Arrest (v1.3) library, were selected. Between two and four siRNAs targeting each of these genes were obtained. The effect of transfecting each of these siRNAs into HeLa cells on TRAIL-induced cytotoxicity was assessed. In total, 11 of the genes had at least one siRNA that had a significant effect on TRAIL-induced cytotoxicity; of these, four had two siRNAs that had a significant effect (Figure 3.13). For each gene, the siRNA that had the greatest effect was selected. These siRNAs were transfected into cells and tested for their effect on TRAIL sensitivity together on one plate. This was compared to the effect of co-transfecting pIRES-P with pSM2 constructs from the Expression Arrest library targeting the same genes, and selecting for puromycin resistant cells, on TRAIL-induced cytotoxicity. In total, nine (TNFSR10A, Caspase 3, PRKRIR, FBXO11, PRKCQ, SMAC, ABL2, BID and Caspase 8) out of the 18 genes tested had an siRNA that significantly increased the survival after TRAIL treatment. In contrast, the only pSM2 construct that had a significant effect on TRAIL-induced cytotoxicity was pSM2.shCasp8.2 (Figure 3.14)

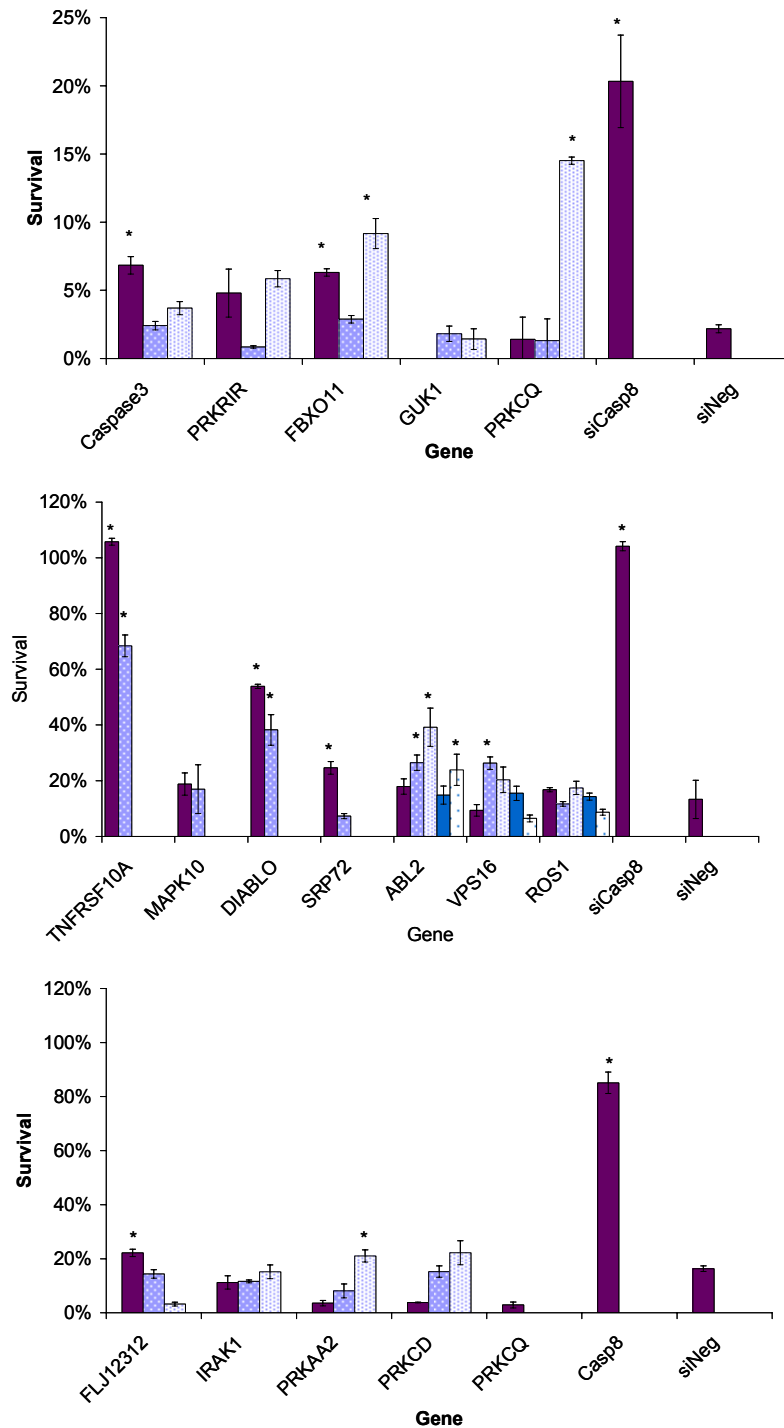


Figure 3.13 Effects of knock-down of 18 positive controls on TRAIL-induced cytotoxicity.

Cells were transfected with between two and four siRNAs targeting 18 genes previously implicated in the TRAIL-induced cytotoxicity pathway, or a non-targeting siRNA (siNeg) on three separate plates. Cells were assessed for sensitivity to 1µg/ml TRAIL 48 hours later. Each shaded bar represents a different siRNA targeting the gene indicated. First results for each of ABL2, VPS16 and ROS1 represent pools of the other four siRNAs for that gene. Results are the mean of three repeats. Error bars represent 1 standard deviation. * results significantly different from negative control using Student's t-test on log transformed data (Bonferroni corrected $\alpha = 0.05$).

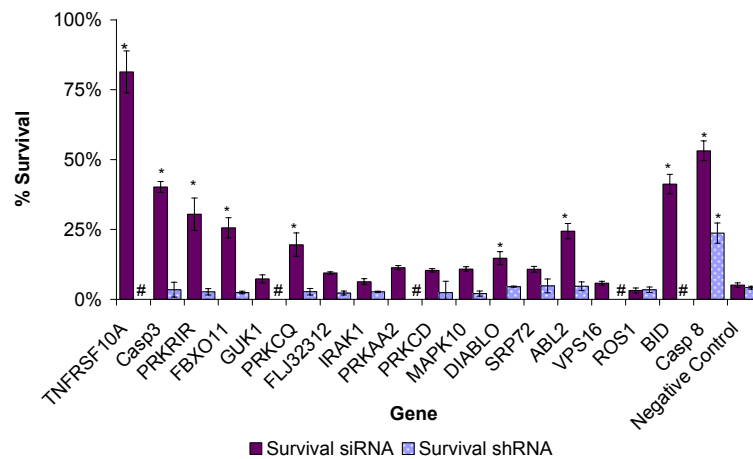


Figure 3.14 Comparison of effect of knock-down of 18 positive controls by siRNA or shRNA^{mir} on TRAIL-induced cytotoxicity.

Cells were either transfected with the best siRNA targeting one of 18 genes previously implicated in TRAIL-induced cytotoxicity or co-transfected with pIRES-P and a vector expressing an shRNA^{mir} targeting the same gene from the Expression Arrest library. siRNA-transfected cells were assessed for TRAIL sensitivity 48 hours later. pIRES-P/shRNA-transfected cells were grown for 24 hours and then selected with 2 μ g/ml for 48 hours before sensitivity to 1 μ g/ml TRAIL was assessed. Data are means of three replicates. Error bars represent 1 standard deviation. * results significantly different from negative control using Student's t-test on log transformed data (Bonferroni corrected $\alpha = 0.05$). # vector did not contain expected hairpin upon sequencing.

In order to find the source of this difference, qRT-PCR was used to measure the change in expression levels of the targeted transcript when siRNA/shRNA^{mir}s were introduced into the cells. Oligonucleotide primers were designed to amplify from the cDNA of each transcript and not from genomic DNA by either including at least one large intron in the amplicon, or spanning an intron/exon boundary with one oligonucleotide primer. Primers were tested to ensure they amplified one and only one fragment and their efficiency measured as described by Pfaffl *et al.* (Pfaffl 2001). Primers were rejected if efficiency was less than 80% or greater than 110% (Pfaffl 2001). Primer pairs that failed were redesigned up to three times. Oligonucleotide primers were successfully designed for 13 genes (see Appendix B). Transfection of siRNAs targeting eight of the 13 genes (61%) caused a reduction in expression levels of the targeted transcript to 30% or less of the level measured when an siRNA was transfected which targets no transcript. Transfection of vectors encoding hairpins targeting three out of 10 genes (30%) led to a similar reduction in transcript levels. There were eight cases where transfection of an siRNA led to a significant reduction in TRAIL-induced cytotoxicity but transfection of a hairpin encoding vector targeting the same gene did not. In all but one of these cases transfection of the siRNA led to a greater reduction in transcript levels than transfection of the hairpin-encoding vector (Figure 3.15).

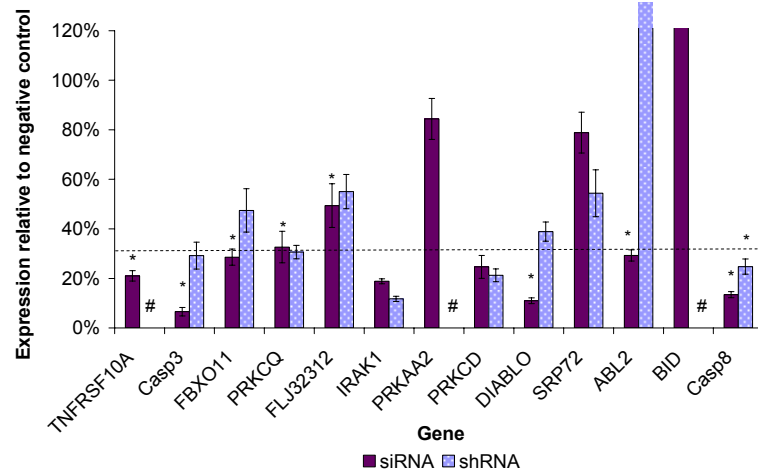


Figure 3.15 qRT-PCR measurement of knock-down by siRNAs/shRNAmirs targeting 13 positive controls

RNA was prepared from cells either transfected with the best siRNAs targeting one of 13 genes previously implicated in TRAIL-induced cytotoxicity or co-transfected with pIRES-P and vectors expressing an shRNA^{mir} targeting the same genes and selected for 48 hours with 2 μ g/ml puromycin. cDNA was produced by reverse transcribing RNA. SYBR green qPCR was carried out using primers designed to amplify a section of the 13 genes targeted, the GAPDH gene and the ACTB gene. Primers were designed to amplify from cDNA only. Expression levels relative to negative controls were calculated using a variation of the Pfaffl method to allow normalization to multiple housekeeping genes using GAPDH and ACTB to normalise samples (Hellemans et al. 2007). * siRNA/shRNA elicited a significant change in TRAIL sensitivity assays. # vector did not contain expected hairpin upon sequencing. . The dashed line represents 30% expression relative to control.

In an attempt to explain the relatively poor performance of the hairpin-encoding vectors, the hairpins used were sequenced. Although the constructs in the expression arrest library are supposed to be sequence-verified, five of the constructs sequenced did not contain the expected hairpin sequence (vectors which were predicted to express hairpins targeted against TNFRSF10A, GUK1, PRKAA2, VPS16 and BID). The results from these genes are not shown in the above results, or included in any calculations or conclusions. Thus in fact shRNA^{mir}s targeting only 13 genes were tested for effects on the TRAIL induced apoptosis, and the effect of only 10 shRNA^{mir}s on levels of the targeted transcripts was measured.

3.5 Conclusion

An assay for the effect of the TRAIL ligand on the viability of HeLa cells has been established. It has been demonstrated that treatment of HeLa cells with the TRAIL ligand leads to cytotoxicity. It has further been demonstrated, by the knock-down of the key TRAIL pathway gene Caspase 8, that TRAIL-induced cytotoxicity can be modulated using both shRNA^{mir}- and siRNA-mediated RNAi against genes involved in the pathway. The optimisation experiments undertaken emphasise the importance of carefully examining the

effect of as many different variables on the outcome of the assay, not only separately but together. For example, decreasing the cell density at the beginning of the assay increases the sensitivity of the cells to TRAIL-induced cytotoxicity (Figure 3.6), but decreases the effect of using RNAi to knock-down genes involved in the TRAIL pathway (Figure 3.9 and Figure 3.8b).

The final assay conditions selected from the optimisation process were to seed 3,000 cells and grow for 24 hours. For siRNA experiments, the cells are transfected with 2.5pmol of siRNA using 0.12µl of Lipofectamine 2000. For shRNA^{mir} experiments, cells are transfected with 80ng of plasmid DNA using 0.24µl of siPort XP-1. Cells are then grown for 48 hours. The viability of cells is assessed using alamarBlue before treatment with 1µg/ml TRAIL in serum-free media for 24 hours. The viability of cells is then assessed again using alamarBlue and the percent survival of the cells is calculated using Equation 6. Using these conditions, 28% of cells transfected with pSM2.shControl and 29% of cells transfected with siGL2 survive TRAIL treatment. In contrast, 51% of cells transfected with pSM2.shCasp8.2 and 96% of cells transfected with siCasp8 survive (Figure 3.11). This compared with an average survival after treatment with TRAIL of 38.5% of negative control-transfected cells reported in previous, similar work (Aza-Blanc et al. 2003), showing that the assay presented here was at least as sensitive as that of Aza-Blanc *et al.*

The effect of using siRNA- and shRNA^{mir}-mediated RNAi against Caspase-8 on the levels of TRAIL-induced cytotoxicity was compared, both by directly comparing the survival rates of cells treated with a range of TRAIL concentrations and using blind pseudo-screens to assess real performance in a screening situation.

In the direct comparison, siRNA-mediated knock-down of Caspase-8 outperformed shRNA^{mir}-mediated knock-down of Caspase-8, with a greater difference between control and Caspase-8 knock-downs being seen using siRNA-mediated knock-down at all concentrations of TRAIL tested. This was true when either the absolute difference or the fold change in survival after TRAIL treatment was considered. One possible reason for this difference could be a difference in transfection efficiency. Co-transfecting with shRNA^{mir}-encoding vectors and a plasmid carrying a puromycin resistance marker and selecting transfected cells using puromycin increased the effective transfection efficiency, as measured by the number of transfected cells compared to the total number of living cells. However, using selection did not improve the absolute difference in TRAIL-induced cytotoxicity between cells transfected with a negative control, and those transfected with a construct targeting Caspase-8, although the fold change in survival between negative control and the Caspase-8 knock-

down was increased. Selection using the marker encoded on the pSM2 vector itself proved not to be effective. The reasons for this remain unknown, although one difference between the puromycin resistance marker on pIRES-P and pSM2 is the promoter used to transcribe the gene, with the marker on pIRES-P being transcribed from a CMV promoter and the marker on the pSM2 vector being transcribed from a PGK promoter.

The differences seen in the direct comparison were reflected in the performance observed in the blind pseudo-screens. Screens performed using shRNA^{mir}s targeting Caspase-8, either selected or unselected, gave less than perfect results with sensitivities of 70% and 77% and false positive rates of 60% and 0% for the selected and unselected protocol, respectively. This reflects the finding from the direct comparisons, where selecting for transfected cells reduced the background noise, but did not increase the absolute size of the positive signal. The Z^2 -factors for these screens were -1.06 and -0.7, respectively. These are both below the minimum Z^2 -factor of 0 thought necessary to perform a successful screen (Zhang, Chung & Oldenburg 1999). In contrast, pseudo-screens performed using siRNAs found 100% of the positive controls with no false positives. Here the Z^2 -factor was 0.42, well above 0 and close to the 0.5 recommended for an “excellent” assay.

The analysis was extended for a further 17 genes previously linked to TRAIL-induced apoptosis. siRNA-mediated knock-down against 50% of these led to a significant reduction in TRAIL-induced cytotoxicity, including all five genes tested involved in the core death pathway (Caspases 8 and 3, the death receptor TNFS1R10A, BID and SMAC). In contrast, shRNA^{mir}-mediated knock-down lead to a significant change in TRAIL-induced apoptosis in only one case: Caspase 8. The failure of several of these vectors can be attributed to the fact that sequencing revealed that they did not contain the expected hairpin sequence, which raises an issue as to the integrity and accurate annotation of the library. However, even when this problem is taken into account, the conclusion that chemically synthesised siRNAs performed better than the shRNA expressing vectors contained in the expression arrest library still holds. One explanation of these results is provided by measuring the levels of targeted transcripts using qRT-PCR. In all but one case, where an siRNA produced a significant reduction in TRAIL-induced cytotoxicity, but the shRNA^{mir} targeting the same gene did not, the siRNA reduced the levels of the transcript further than the shRNA^{mir}.

. Taken together, these results show, that in the context of a screen for genes that alter the sensitivity of cells to TRAIL-induced apoptosis, the commercially designed and purchased siRNAs are more powerful than the shRNA^{mir}s expressed from the clones in the Expression Arrest library. Not only do the blind pseudo-screen results demonstrate that

shRNA^{mir}-mediated knock-down of Caspase 8 does not provide a large and reliable enough difference from control transfected cells to allow a large-scale screen, but knock-down of no other gene using constructs from the Expression arrest library gives a significant change in TRAIL-induced cytotoxicity. The reasons for this, beyond the fact that the shRNA^{mir}-encoding vectors do not induce such a large reduction in transcript levels, remain unknown. There are three possible reasons why this might be the case. Firstly, the shRNA^{mir} may not be expressed at a high enough level in a large enough number of cells. Secondly, they may not be being properly processed to give active siRNAs. Thirdly, the shRNA^{mir}s are being expressed and processed, but are not inducing degradation of the targeted mRNA. In the first case, the fact that selecting for transfectants does not eliminate the difference suggests that the raw transfection efficiency is not the reason behind the differences. However, this does not rule out the amount of plasmid being delivered or the level of transcription from the transfected plasmid being the cause of the difference in performance. In the third case, it should be noted that the sequence of the shRNA^{mir} and the siRNAs were not generated using the same algorithm. In this case, it would not simply be a case of shRNA^{mir}s being less powerful than siRNAs, but one of these shRNA^{mir}s being less powerful than these siRNAs.

Screens may be performed either in a clone-by-clone manner, with each well on a 96- or 384-well plate transfected with one or multiple siRNA(s)/shRNA^{mir}-encoding vector(s) targeting one gene, or in a pooled manner, where hairpin-encoding vectors targeting multiple genes are transfected into a population of cells. A selection is applied to the population of cells and the shRNA^{mir}s present in the surviving cells are determined, based on the assumption that shRNA^{mir}s that allow cells to escape the selection will be over-represented in the post selection population. Such a strategy could reduce the cost and increase the throughput of screening. In an ideal assay, the selection applied would be strong enough to allow only cells expressing hairpins targeting genes involved in the process in question to escape. However, this is not the case for in the assay as optimised here with a relatively large number of negative control transfected cells surviving treatment with TRAIL. Together with the results on the poor performance of shRNA^{mir}s, this suggests that adopting a pooled screen strategy would be neither efficient nor successful in this case. It should also be noted that preparing transfection quality DNA from the full library would be an expensive and time consuming process (eliminating one of the supposed benefits of shRNA^{mir} based approaches – cost). Therefore, at least in the context of the assay for TRAIL-induced cytotoxicity presented here, screens should be performed using libraries of arrayed siRNAs rather than shRNA^{mir} expressing vectors.

4 AN siRNA SCREEN OF 897 KINASES AND PHOSPHATASES

In Chapter 3 an assay for sensitivity to TRAIL-induced apoptosis was developed, and this assay was used to compare different methodologies for conducting an RNAi screen for genes involved in the TRAIL-induced apoptosis pathway. It was concluded that in this system a gene-by-gene siRNA screen is the most powerful approach. In this chapter these findings are applied to a screen of 897 genes classified as either protein kinases, protein

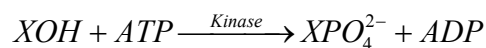
phosphatases or kinase/phosphatase associated. This screen serves both as a gene discovery experiment in its own right and as a pilot for possible larger screens. A rigorous confirmation strategy is employed to eliminate false positives. Attempts are made to define the point in the apoptotic pathway at which they act and the specificity of their regulation of apoptotic pathways is examined.

4.1 Introduction

4.1.1 Kinases and Phosphatases

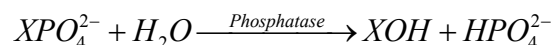
The cell is a highly complex ordered system which depends on the correct regulation of the protein components of cellular pathways. The activity of proteins can be altered in many ways, but one of the most common is by the addition and removal of phosphate groups. Phosphate groups can alter the activity of proteins in several ways. Firstly, the process of phosphorylation has a large free energy. As such phosphorylation can radically alter the preferred conformational state of a protein. Secondly, the phosphate group carries two electrical charges and can accept three hydrogen bonds, which can alter the strength and specificity of the binding of the protein to other proteins and to substrates.

The phosphorylation state of proteins is controlled by kinases and phosphatases. Kinases catalyse the transfer of phosphate from a mono-nucleotide (usually ATP) to specific serine, threonine or tyrosine residues in the following, essentially irreversible, reaction:



Kinases are the largest protein family in the genome and contain a characteristic 250 amino acid kinase domain. They are involved in many aspects of cellular regulation including control of the cell-cycle and mitogen-activated kinase signalling pathways.

The dephosphorylation of proteins is catalysed by protein phosphatases, which catalyse the removal of phosphate groups from protein in the following, again irreversible, reaction:



Note that these two activities are not quite the opposite of each other, with the overall reaction of $ATP + H_2O \rightarrow ADP + HPO_4^{2-}$ effectively consuming one ATP molecule. There are fewer phosphatases in the genome than there are kinases, and the specificity of phosphatases is often regulated by phosphatase regulatory subunits.

While information in the apoptotic pathway is usually passed by proteolysis rather

than phosphorylation, many kinases have been implicated in regulation of apoptotic pathways in general and the TRAIL-induced apoptotic pathway in particular (see section 1.3.2.6). Indeed the Aza-Blanc screen concentrated mainly on kinases (Aza-Blanc et al. 2003). Repeating this should allow of an investigation of the reproducibility of results from RNAi screens. The involvement of kinases in the regulation of apoptotic pathways would also imply the involvement of phosphatases and their regulatory subunits.

4.1.2 The Library

The library used in this chapter is the Qiagen Kinase/Phosphatase library, which is a subset of the Qiagen Druggable Genome library v2. The library contains siRNAs targeting 691 kinases (there are 805 genes listed in Ensembl 46 associated with the GO term “Kinase activity”) and 206 phosphatases and phosphatase associated proteins (159 genes associated with the GO term “Protein phosphatase activity” and 59 with “Protein phosphatase regulatory activity”). Each gene is targeted by 2 siRNAs designed using a neural network based algorithm (Huesken et al. 2005) and checked for specificity using a proprietary similarity based algorithm. The library is supplied on 24 96-well plates with one siRNA per well. Each plate contains 80 sample siRNAs and 2 non-targeting controls, a scrambled siRNA (QiaNeg) and an siRNA targeting GFP (siGFP), leaving 14 empty wells which can be used for controls.

4.1.3 Analysis of screening data

The aim of the analysis pipeline for siRNA screening data is to take raw assay outputs and produce a single score for each siRNA. The simplest analysis schemes calculate the number of standard deviations the average value of a well is from the mean value for the screen. A more comprehensive scheme has been proposed by Boutros *et al* and implemented in the R/Bioconductor package cellHTS. There are five steps from raw data to final score (Boutros, Brás & Huber 2006) :

1. **Production of a metric for each well.** Here that metric is survival calculated as described in Equation 6.
2. **Transformation of data.** A transformation such as a logarithmic transformation may be applied to the data.
3. **Plate normalisation.** As seen in Figure 3.13, data often shows plate to plate variation, and therefore data must be normalized before results from different plates can be directly compared.
4. **Standardisation.** Scores are standardized to produce a z score for each well. This

can be done using the mean and standard deviation, or using the median and median absolute deviation.

- 5. Summary of replicates.** A function must be applied to produce one score from a number of screen replicates.

There are several different alternatives for plate normalization. Data maybe normalized using the values of the negative controls on each plate. A related method to this is the normalized percentage inhibition which rescales data so that the negative control has a value of 0 and the positive control a value of 1:

$$NPI(x) = \frac{x - \mu_{negatives}}{\mu_{positives} - \mu_{negatives}} \times 100\%$$

Equation 1

where: $\mu_{positives}$ is the geometric mean of the positives controls and $\mu_{negatives}$ is the geometric mean of the negative controls.

This method makes no assumption about the effect size of the samples, but is very sensitive to variance in the control wells. If there is an assumption that only a small number of siRNAs on each plate are “hits”, then data maybe normalized to some measure of the central tendency of the sample data, such as the mean or median of samples on the plate. If the median is used, this method provides normalization that is robust with respect to small numbers of outliers. It does however rely on only a small number of siRNAs on each plate eliciting a strong phenotype. One situation in which this may not be the case is if the arraying of genes within the library is not random.

Different functions can also be applied to summarize the replicates. The most common are the mean, the minimum and the maximum. The use of the minimum is the most conservative summary. It asks the question: is the score of this siRNA high in every replicate? The maximum is the least conservative summary; it asks the question: is the score of this siRNA high in any of the replicates? The mean is between these two extremes. Other options include the calculation of p values using t-tests if there is sufficient data.

The cellHTS package provides a frame-work for carrying out each of these steps, calculates plate and screen level quality controls. Results are presented as a single R object and as a series of linked HTML reports.

4.2 Screen design, execution and data analysis

In order to screen the library of siRNAs for genes that affect the TRAIL-induced apoptosis pathway, aliquots of siRNAs from the library plates were transferred to new plates and transfected into HeLa cells. In order to reduce variability all cells used had been passaged exactly four times after defrosting. To each plate of siRNAs the following controls were added (Figure 4.1):

- **siNeg, QiaNeg, and siGFP.** Assay negative controls. These controls were designed not to target any gene which is expressed in human cells.
- **siCasp8, siBID, siSMAC.** Assay positive controls. These controls are siRNAs known to reduce the sensitivity of HeLa cells to TRAIL-induced apoptosis.
- **siKIF11.** RNAi positive control. siKIF11 induces growth arrest and cell death upon transfection. This allows that extent of the success in transfecting cells and inducing RNAi to be estimated.
- **No transfection.** Transfection negatives. Allows assessment of the effect of transfection on the assay
- **Blank.** Assay blanking wells. These wells contain no cells and allow for the assay to be zeroed.

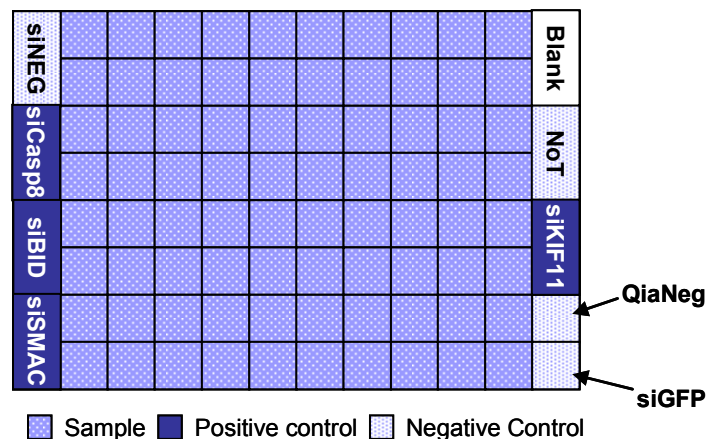


Figure 4.1 Plate layout used in screen

Plates were transfected in batches, and grown for 48 hours. Viability was assessed using alamarBlue. Cells were then treated with 0.25 μ g/ml TRAIL for 24 hours before viability was reassessed. Throughput was increased as the screen progressed, starting at six plates per day and reaching a maximum throughput of 24 plates per day.

The “quality” of the results from a screening plate can be judged by the dynamic range of the positive and negative controls, defined as the ratio of the geometric means of

the survival in siCasp8 transfected wells to the geometric mean survival of negative control transfected wells. A dynamic range of 2 means that the average survival in siCasp8 transfected wells is twice that in negative control wells. A quality control threshold for acceptable plates was arbitrarily set at a dynamic range of 2. The initial design required three replicates of the screen to be completed. After the completion of 2.75 replicates of the screen it was observed that the dynamic range of plates from the first of the replicates was lower than that found in plates from the other two replicates (Figure 4.2). The percentage of plates failing this quality control criterion for each replicate was 44%, 4% and 4% respectively. In all but two cases the quality of each plate was higher in replicates two and three than in replicate one. Including data from replicate one would therefore reduce the overall quality of data. On this basis it was decided to combine the three replicates to produce two “high-quality” replicates. Data from the first replicate were discarded, except in those cases where the dynamic range between siCasp8 and negative control transfected wells was less than 2 in one or other of the two remaining plates, and the plate from the first replicate had a higher dynamic range. In this case the plate with a dynamic range of less than 2 was discarded and replaced with the plate from the first replicate. In this way the screen simulated a situation where the screen was performed in duplicate, and plates failing a quality control measure were repeated. Only 4% of plates in the resulting two replicates had dynamic ranges of less than 2.

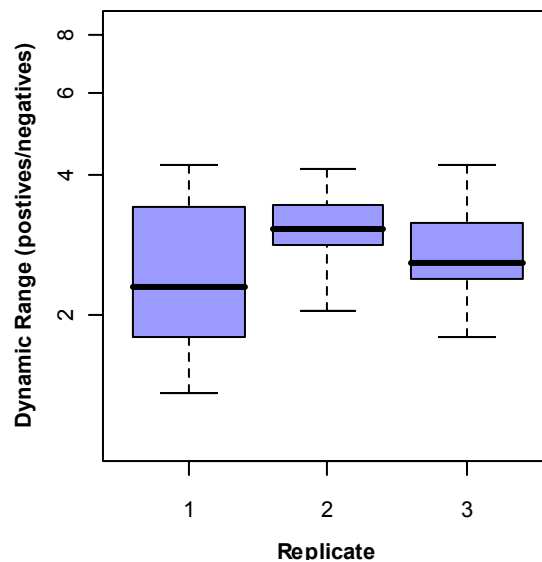


Figure 4.2 Boxplot of dynamic ranges of plates from different screen replicates

The dynamic range of results from each plate was calculated as the ratio of the geometric mean of survival in siCasp8 transfected wells to the geometric mean of survival in negative control transfected wells. Plates were grouped by the replicate of the screen from which they originated. $n = 18, 24$ and 24 for the first, second and third replicates respectively.

In order to investigate the effects of different plate normalization techniques, unnormalized data (Figure 4.3a) was compared to data normalized using the geometric mean of the plate negative controls (Figure 4.3b), the median of the survivals for the samples on each plate (Figure 4.3c) and using normalized percentage inhibition (Figure 4.3d). Examination of these plots indicates that of the different normalisation methods median normalization produces the most consistent results, with greater consistency in the position of the quartiles as well as the measures of centre. Data normalized using the geometric mean of the negative controls is little different from unnormalized data, while data normalized using NPI is more consistent, but has a few very obvious outliers.

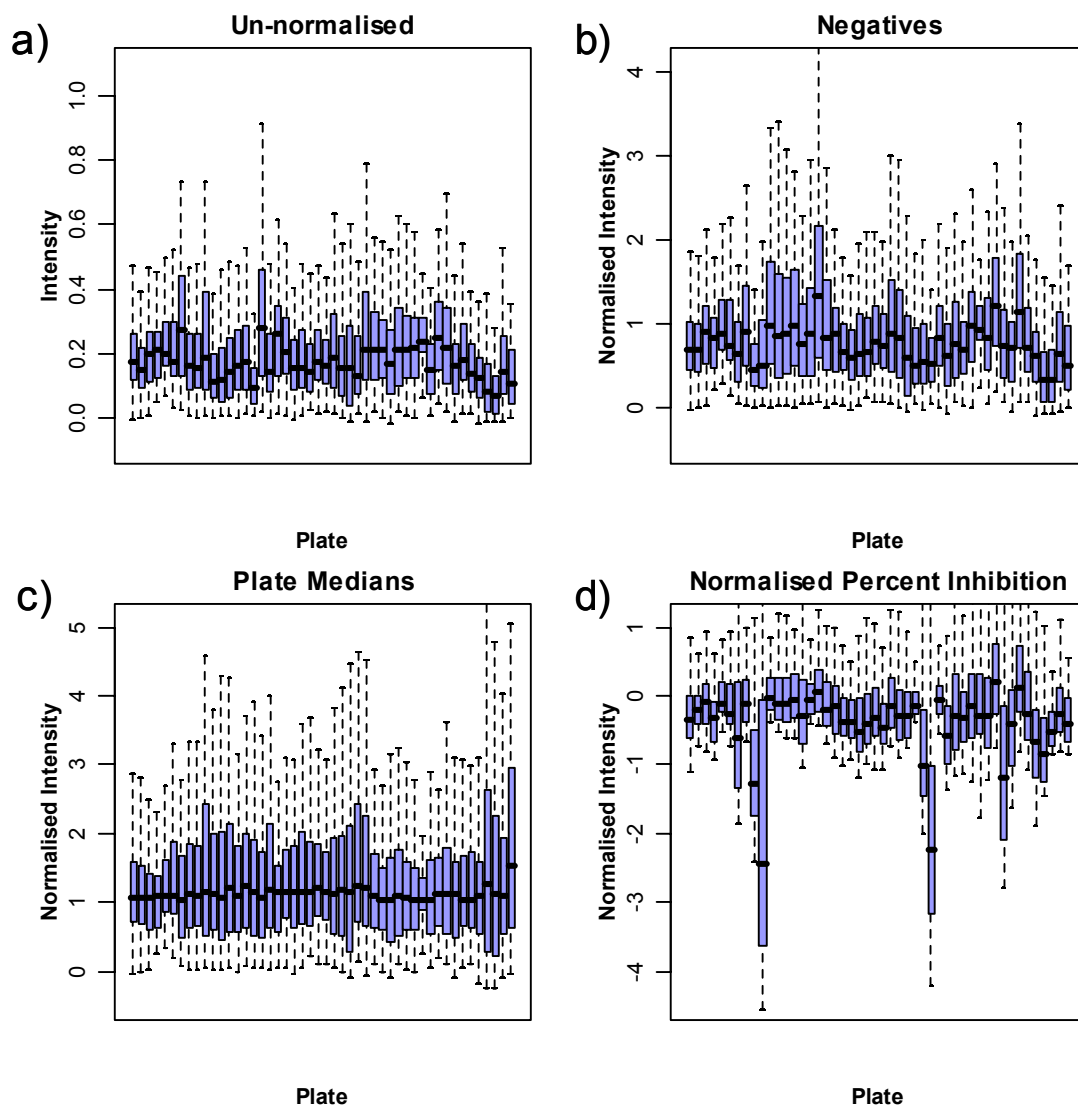


Figure 4.3 Normalization of data from screen of Kinases and phosphatases.

Boxplots showing values for each plate from a) un-normalised data, b) data normalized by dividing values by the geometric mean of negative controls, c) data normalized by dividing by the median of samples of each plate and d) data normalised using NPI as defined in Equation 1.

A strong relationship between effect size and variance indicates non-normally distributed data. If there is a strong relationship between effect size and variance then this

may be reduced by applying a logarithmic transformation to the data. To investigate whether a log transformation would improve the interpretability of data here, the rank of the mean of the normalized values for each siRNA was plotted against the standard deviation between replicates (Figure 4.4). A clear relationship can be observed between the mean and the standard deviation in both non-transformed and log transformed data. In non-transformed data the standard deviation increases as the mean increases (Figure 4.4a). This is the expected behaviour for ratio data. Log transforming the data reverses the trend with smaller means having larger standard deviations than larger means (Figure 4.4b). The running median line, shown in red, should be flat if there is no relationship between the mean and the standard deviation. The deviation of the line from flat indicates the strength of the relationship. Comparing the running median line for non-transformed data and log-transformed data leads to the conclusion that the strength of the relationship is not reduced, and may even be increased by log transformation of the data; therefore data was used untransformed in further analyses. However, this means that data cannot be treated as normal and so the normal distribution cannot be used to determine p values representing the probability that any given siRNA affects the sensitivity of cells to TRAIL-induced cytotoxicity, when compared to the distribution of siRNA effects.

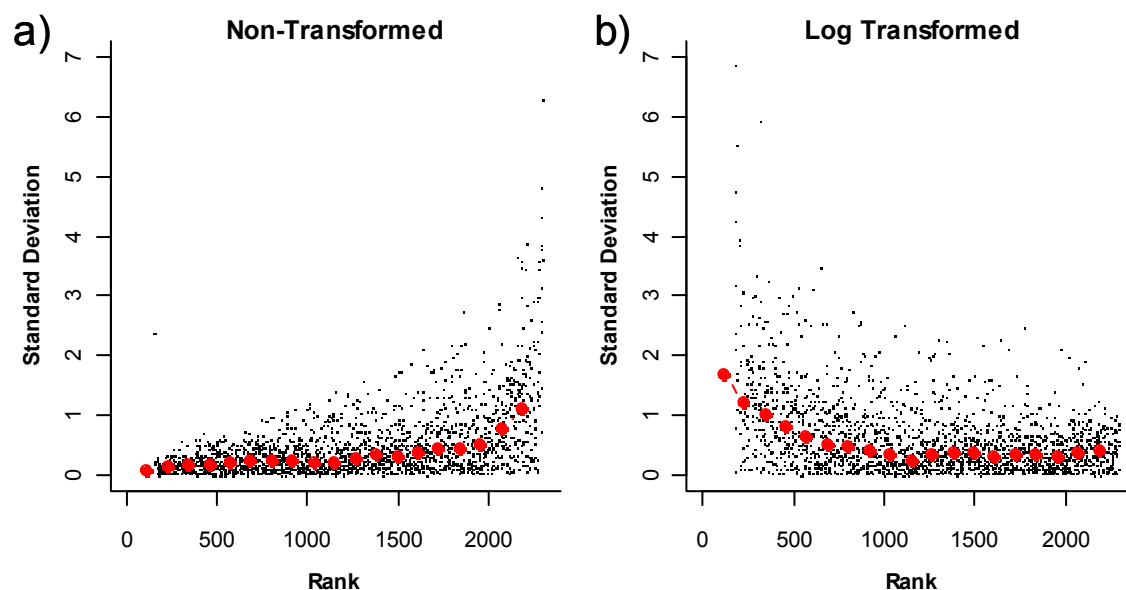


Figure 4.4 Relationship between standard deviation and rank of the mean for siRNAs.

For each siRNA the mean of the normalized data for each replicate was calculated. The rank of this mean was then plotted against the standard deviation between the replicates for a) Non-transformed data and b) Transformed data. The red line in each plot represents the running median standard deviation.

Given that only two replicates of the screen were conducted, it was decided to use the minimum of the replicates to summarise the data. This means that a high final score represents an siRNA which has large effect which repeated in both replicates.

Data was processed using the R/Bioconductor package cellHTS using a median

normalization without log transformation and the minimum as a summary function. The HTML report produced can be found on the CD accompanying this work or at web address http://www.sanger.ac.uk/HGP/Chr22/RNAi/TRAIL_Kinase/.

4.3 Screen quality and analysis of controls

The processed screening data was used to assess the quality of the screen (Figure 4.5). The Pearson's correlation co-efficient between the two repeats of the screen is 0.65 and spearman's $\rho = 0.66$. Both correspond to a p value of less than 2.2×10^{-16} , showing that this is a real correlation. The replicates are less well correlated at higher scores than at lower scores (Figure 4.5a), this is expected from the finding that variance increases with increasing mean (Figure 4.4a). The correlation between two siRNAs targeting the same gene is much weaker (Figure 4.5b), with a Pearson's correlation co-efficient of 0.2 and a spearman's ρ of 0.17. The weakness of this correlation suggests that while the effect of a particular siRNA on the sensitivity of cells to TRAIL-induced cytotoxicity is fairly reproducible, the effect of different siRNAs targeting the same gene is not. There are two possible explanations for this: the effectiveness of siRNA A and siRNA B at knocking down the targeted gene could vary, or alternatively siRNA A and siRNA B have a number of different off-target effects.

Figure 4.5c shows the distribution of plate dynamic ranges (ratio of the geometric mean of the siCasp8 transfected wells to the geometric mean of the negative control transfected wells). The majority of plates (83%) have a dynamic range between 2 and 4, and only two plates (4%) have a dynamic range of less than 2.

Figure 4.5d shows the distributions of scores for different well types and some of the same data is summarized in Table 4-1. The median score for all positive controls is greater than either the negative controls or samples. As expected from data in Chapter 3 the size of the difference between the negative control transfected wells and siBID or siSMAC transfected wells is smaller than the difference between negative controls transfected wells and siCasp8 transfected wells. The spread of results for siCasp8 is larger than that for other controls, possibly due to the relationship between mean and variance (Figure 4.4a). The Z' factors for comparing siCasp8, siBID and siSMAC to the negative controls are -0.35, -1.05 and -4.08. This is a large reduction on Z' -factor seen for siCasp8 in the previous chapter, although it is close to 0 when compared to the Z' -factors for siBID and siSMAC. In the case of siSMAC it is clear that even though the median score and survival (0.86 and 28.51% respectively) are higher than those for the negative controls (0.27 and 23% respectively) there is little chance of separating the individual values of negative control transfected wells and

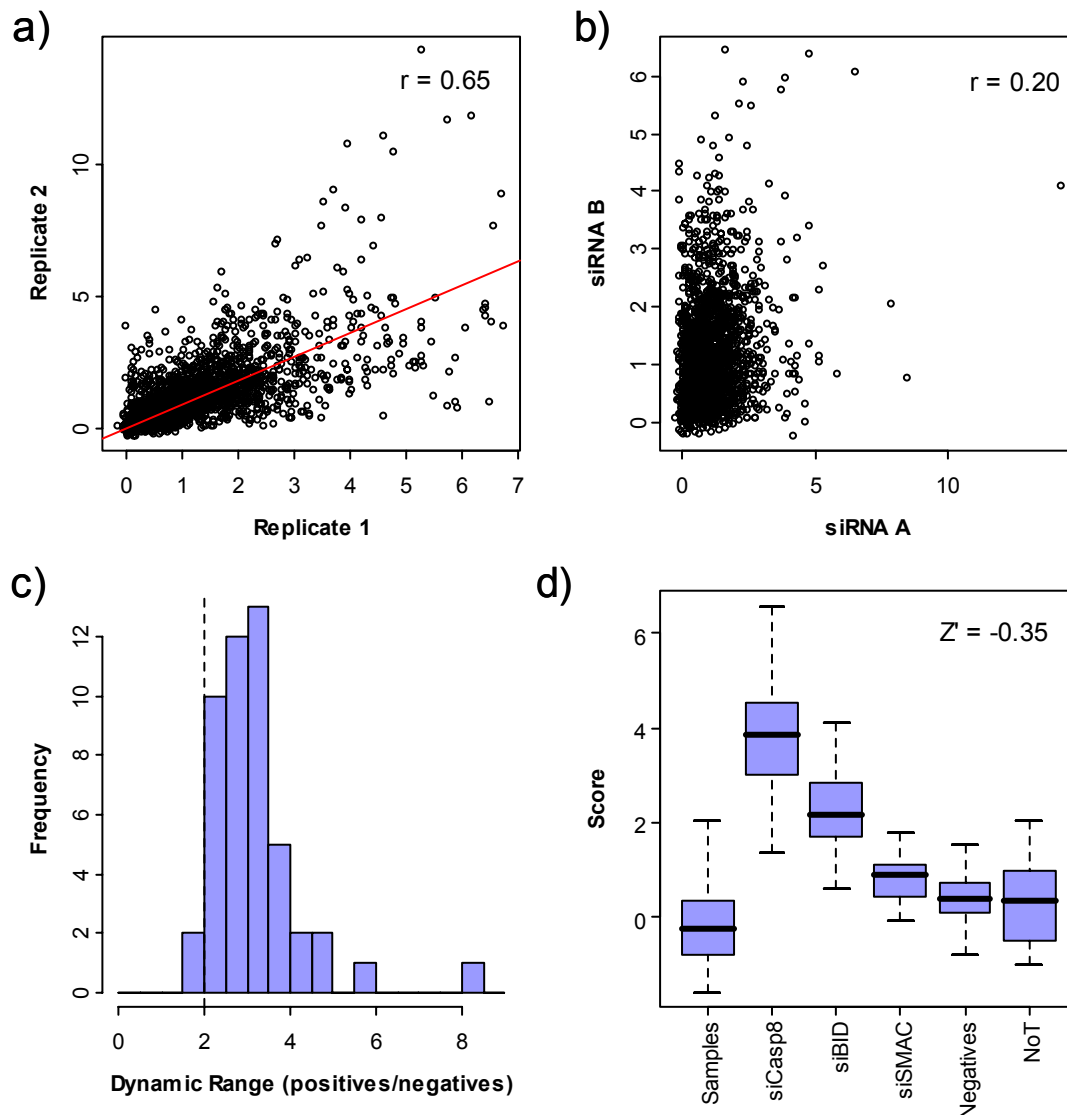


Figure 4.5 Assessment of screen quality and controls.

a) Normalized survival from replicate 1 plotted against normalised survival from replicate 2. Red line shows linear regression of replicate 2 on replicate 1. The Pearson's correlation co-efficient is shown in the top right corner. b) Plot showing normalised survival of the two siRNAs targeting the same gene. The Pearson's correlation co-efficient is shown in the top right corner. c) Histogram showing the distribution of plate dynamic range (see above for definition of plate dynamic range). Dashed line represents a dynamic range of 2. d) Box plot summarising the scores in different well types. Z' -factor between negative controls and siCasp8 is shown in top right corner. NoT = Untransfected

siSMAC transfected wells. This analysis of the positive controls shows that while the screen may be sensitive enough to pick up siRNAs with an effect as strong that of siCasp8, more subtle effects, such as those elicited by siSMAC are likely to be missed.

The scores and survivals of the non-transfected wells (0.32 and 24.9% respectively) are similar to those for the negative controls although the non-transfected wells have a higher spread of values (Figure 4.5d and Table 4-1). The median value of the samples is slightly reduced compared with the negative controls (-0.26 and 15.20% median score and median survival respectively), suggesting that either the library contains some siRNAs that actively increase sensitivity to TRAIL-induced cytotoxicity, or that one of the negative

<i>Category</i>	<i>Median Score</i>	<i>Median Survival</i>	<i>MAD Survival</i>
Samples	-0.26	15.20%	12.20%
siCasp8	3.788	64.00%	16.37%
siBID	2.2	48.32%	8.44%
siSMAC	0.86	28.51%	6.66%
Negatives	0.27	23%	8.47%
Untransfected	0.32	24.90%	16.18%

Table 4-1 Summary statistics of controls in Kinase and Phosphatase screen.

The median score, median survival and the median absolute deviation (MAD) of survival is shown

controls is triggering an off-target effect that is reducing the sensitivity to TRAIL-induced cytotoxicity.

4.4 Screen results

The results of the screen, in the form of single scores for each siRNA, are summarized in Figure 4.6. The distribution of scores has a long right hand tail and a foreshortened left hand tail (Figure 4.6a) compared to a normal distribution. The long right hand tail represents siRNAs that have reduced the sensitivity of cells to TRAIL. The foreshortened left hand tail is probably due to the lower limit of the measurement used i.e. it is not possible for less than 0% of cells to survive.

In order to further examine the distribution of the screening results, the rank of siRNA scores was plotted against the score for that siRNA (Figure 4.6b). The distribution of scores is continuous and the rate of increase in score with rank is constant for a large portion of the plot. The scores from this portion of the ranked list cover a large proportion of the observed range of scores. This implies that siRNA cannot be divided into two distinct classes: those that have an effect on sensitivity to TRAIL-induced cytotoxicity and those that do not have an effect, but that each siRNA has a more or less strong effect on the sensitivity of cells to TRAIL.

Figure 4.6c shows the spatial distribution of scores within the library in order to test for plate position effects. High and low scores are relatively evenly distributed between and within plates and there are not obvious signs of edge effects.

siRNAs were ranked by their score in the screen. A portion of the resulting table is shown in Table 4-2. The complete table is available as part of the screen report, on the included CD or online at http://www.sanger.ac.uk/HGP/Chr22/RNAi/TRAIL_Kinase/. The results of the screen were also summarized on a gene-by-gene basis. A portion of this summary is shown in Table 4-3.

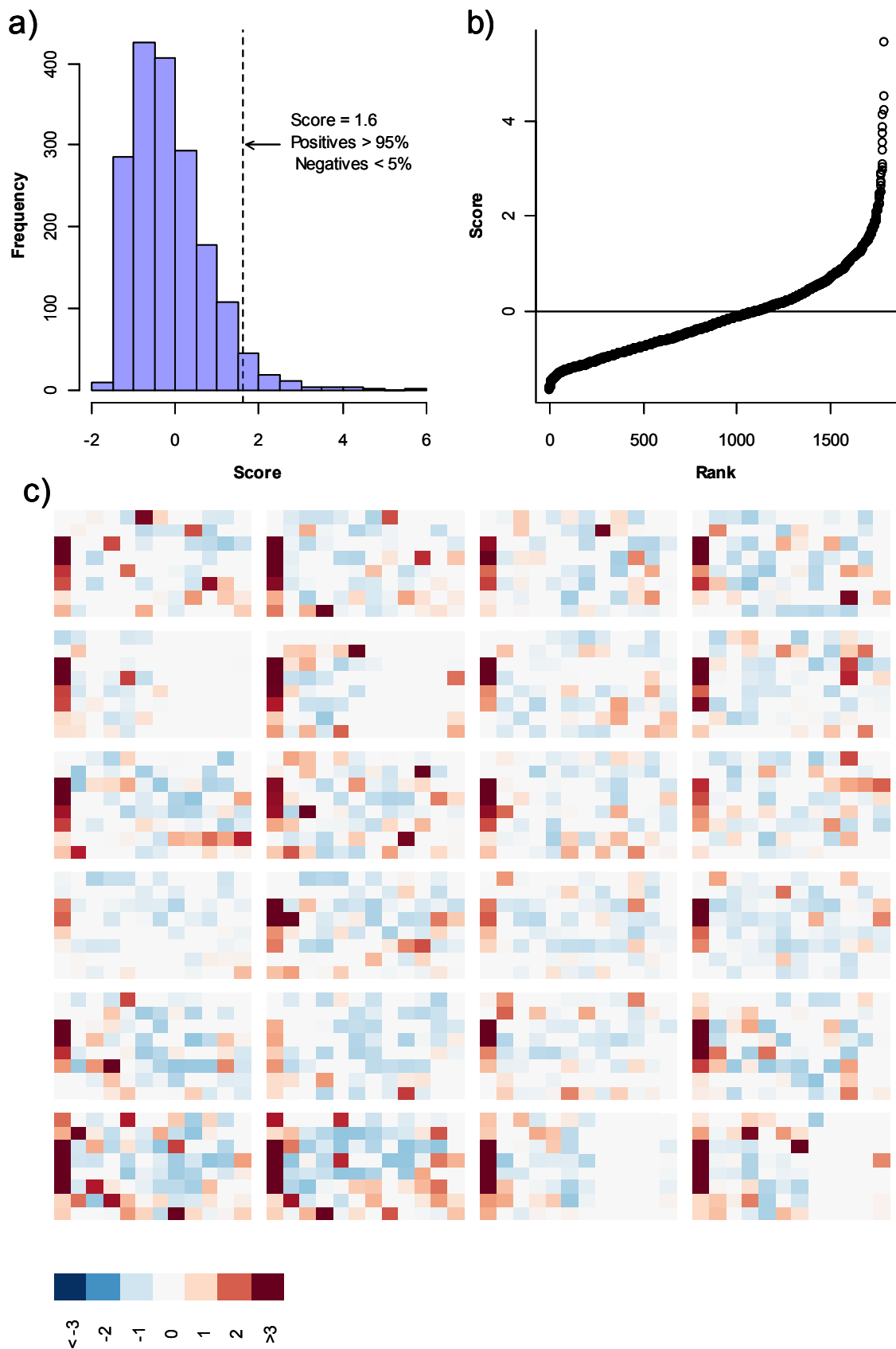


Figure 4.6 Results of siRNA screen of Kinases and Phosphatase

a) Histogram of scores from sample wells. Dashed line represents a score of 1.6. b) Rank of siRNA score plotted against score. c) Heat map of scores per plate. siRNAs with a highly positive score are shown in red, siRNAs with a highly negative score are shown in blue. Plates are arranged row wise

RefSeq ID	Symbol	Description	Normalized Survival		score
			Rep 1	Rep 2	
NM_030974	Sharpin	shank-interacting protein-like 1	5.3	14.2	5.46
NM_005541	INPP5D	inositol polyphosphate-5-phosphatase, 145kDa	4.8	4.9	4.81
NM_001396	DYRK1A	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A	4.6	4.2	4.21
NM_018401	STK32B	serine/threonine kinase 32B	4.2	7.9	4.10
NM_198828	LOC375449	similar to microtubule associated testis specific serine/threonine protein kinase	4.0	4.3	3.79
NM_005399	PRKAB2	protein kinase, AMP-activated, beta 2 non-catalytic subunit	6.1	3.8	3.65
NM_014002	IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	3.7	4.1	3.46
XM_086287	PTPRV	protein tyrosine phosphatase, receptor type, V	3.6	3.8	3.31
NM_020791	TAOK1	TAO kinase 1	4.2	3.5	3.28
NM_007079	PTP4A3	protein tyrosine phosphatase type IVA, member 3	3.6	8.5	3.24
NM_006240	PPEF1	protein phosphatase, EF hand calcium-binding domain 1	3.5	5.2	3.21
NM_001570	IRAK2	interleukin-1 receptor-associated kinase 2	3.7	3.4	3.08
NM_002827	PTPN1	protein tyrosine phosphatase, non-receptor type 1	3.4	4.3	3.04
NM_001556	IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	5.5	3.3	2.98
NM_016086	DUSP24	dual specificity phosphatase 24 (putative)	3.3	6.4	2.88
NM_173354	SNF1LK	SNF1-like kinase	3.2	3.7	2.83
NM_018638	ETNK1	ethanolamine kinase 1	3.1	6.4	2.69
AB033076	KIDINS220	likely homolog of rat kinase D-interacting substance of 220 kDa	3.0	3.9	2.59
NM_004577	PSPH	phosphoserine phosphatase	3.0	3.3	2.57
NM_022128	RBKS	ribokinase	3.0	3.0	2.49

Table 4-2 Top scoring siRNAs from an siRNA screen of Kinases and Phosphatases.

Table shows top 20 scoring siRNAs from the screen. The complete table is available on the included CD or online at http://www.sanger.ac.uk/HGP/Chr22/RNAi/TRAIL_Kinase

<i>RefSeq ID</i>	<i>Gene Symbol</i>	<i>Description</i>	Score siRNA A	Score siRNA B	Minimum Score	Maximum Score	Mean Score
NM_020836	KIAA1446	Brain-enriched guanylate kinase-associated protein	2.42	2.45	2.42	2.45	2.44
NM_000788	DCK	Deoxycytidine kinase	2.46	2.15	2.15	2.46	2.30
NM_030974	Sharnin	Shank-interacting protein-like 1	5.46	2.15	2.15	5.46	3.81
NM_018638	ETNK1	Ethanolamine kinase 1	1.81	2.69	1.81	2.69	2.25
NM_021132	PPP3CB	Protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform (calcineurin A beta)	1.82	1.65	1.65	1.82	1.74
NM_173354	SNF1LK	SNF1-like kinase	1.42	2.83	1.42	2.83	2.13
NM_018401	STK32B	Serine/threonine kinase 32B	4.10	1.35	1.35	4.10	2.72
NM_001896	CSNK2A2	Casein kinase 2, alpha prime polypeptide	1.32	1.81	1.32	1.81	1.57
NM_001556	IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	1.22	2.98	1.22	2.98	2.10
NM_183048	PRKCBP1	Protein kinase C binding protein 1	1.48	1.21	1.21	1.48	1.35
NM_033118	MYLK2	Myosin light chain kinase 2, skeletal muscle	1.21	1.45	1.21	1.45	1.33
NM_145203	CSNK1A1L	Casein kinase 1, alpha 1-like	1.44	1.18	1.18	1.44	1.31
NM_017823	DUSP23	Dual specificity phosphatase 23	1.66	1.17	1.17	1.66	1.42
NM_019892	INPP5E	Inositol polyphosphate-5-phosphatase, 72 kda	1.17	1.09	1.09	1.17	1.13
NM_007064	TRAD	Serine/threonine kinase with Dbl- and pleckstrin homology domains	1.07	2.21	1.07	2.21	1.64
NM_003558	PIP5K1B	Phosphatidylinositol-4-phosphate 5-kinase, type I, beta	1.07	1.94	1.07	1.94	1.50
NM_014683	ULK2	Unc-51-like kinase 2 (C. Elegans)	1.06	1.52	1.06	1.52	1.29
NM_004443	EPHB3	EPH receptor B3	1.04	1.09	1.04	1.09	1.07
NM_001798	CDK2	Cyclin-dependent kinase 2	1.10	0.99	0.99	1.10	1.04
NM_020639	RIPK4	Receptor-interacting serine-threonine kinase 4	0.94	1.58	0.94	1.58	1.26

Table 4-3 Extract from table summarizing screen results on a per gene basis.

Genes are ranked on the basis of the minimum of the scores from the two siRNAs. Full table is available on included CD or online at http://www.sanger.ac.uk/HGP/Chr22/RNAi/TRAIL_Kinase/perGene.tab

4.4.1 Hit selection

The continuous distribution of scores makes distinction of “hit” genes from the negative genes difficult (and to a certain extent meaningless). However, in order to select genes for confirmation and follow up it is necessary to apply a cut off. A cut off was selected on the basis of the distributions of the siCasp8 positive control transfected wells and negative control transfected wells. In order to maximise the number of potential hits selected from this first pass screen, a cut-off was selected such that 95% of siCasp8 transfected wells score higher than the threshold. A cut off score of 1.6 selects 95% of siCasp8 transfected wells, but only 5% of negative control transfected wells (Figure 4.6a). 71 siRNAs targeting 66 genes have a score of greater than 1.6. In this way these siRNAs are identified as ‘hit’ siRNAs.

It is important to note that identifying an siRNA as a hit siRNA does not necessarily identify the gene it is targeted by as a hit gene. This is due to the possibility that the effect of the siRNA maybe due to off-target effects.

One possibility for selecting hit ‘genes’ is to look for genes where both siRNAs targeting the gene are hits – that is where both siRNAs score higher than 1.6. This method selects 5 genes as candidate hit genes for confirmation and follow-up. A second possibility for selecting hits for follow-up would be take genes targeted by a single scoring siRNA. Using a similar method for selecting a cut as was used above would make this a less conservative method for selecting hits. However, limitations on time and resource would make selecting all 66 genes targeted by a single siRNA scoring higher than 1.6 unfeasible. Therefore a smaller number of genes must be selected for confirmation and follow-up depending on the resources available. Here genes targeted by the top ten scoring siRNAs were selected for confirmation and follow-up as it was assumed that these were the genes most likely to confirm. Unfortunately, while this method does allow for a definitive answer for the involvement these ten genes, it offers no information on the other 55 genes targeted by the remaining 61 siRNAs: i.e. it is incorrect to say that they are not hit, simply that they are untested.

It is possible that where an siRNA scores below zero, transfection of the siRNAs maybe result in an increase in the sensitivity of cells to TRAIL-induced cytotoxicity. It might be interesting to follow-up some of these genes. However, the assay and the data analysis protocol was designed and optimised to find siRNAs which decrease the sensitivity of cells to TRAIL. Given limited resources it was decided that following up the hits that the screen

was designed to find was a more likely to yield interesting results, and so siRNAs that appear to increase sensitivity to TRAIL were not further investigated.

4.4.2 Analysis of genes previously associated with the TRAIL pathway.

The performance of siRNAs targeting genes previously associated with the TRAIL pathway provides a means of assessing the sensitivity of the screen. The results from the kinase and phosphatase screen conducted here for siRNAs targeting genes previously associated with the TRAIL pathway in the Aza-blanc *et al* screen (Aza-Blanc et al. 2003) and in other literature, are shown in Table 4-4. The Aza-blanc screen included siRNAs targeting 510 genes including 360 known and predicted kinases. The remaining genes were hand picked ‘genes of interest’. The screen here screened siRNA targeted 691 kinases and 206 phosphatases. While the complete list of genes targeted by Aza-blanc *et al* is not available, it can be assumed that the majority of the 360 kinases screened in Aza-blanc overlap with the set screened here, while the remaining 150 do not. Of the 20 genes designated hits in the Aza-blanc screen, 14 were also targeted in the screen presented here. Only one siRNA targeting genes which reduced the sensitivity of cells to TRAIL-induced cytotoxicity in Aza-blanc *et al* or elsewhere in the literature has a score of greater than 1.6. One siRNA targeting ROS1 scored 1.7 and is ranked 65. The next highest scoring siRNA is an siRNA targeting GSK3 α which scores 1.52 and is ranked 81. Thus using the first criteria for a hit – that both siRNAs targeting a gene must be about 1.6, none of the genes previously identified count as a hit. Under the second criteria for a candidate hit – that only one siRNA must score above 1.6, one gene counts as a hit, although none of the previously identified genes are within the 10 genes selected for confirmation based on this criteria. This gives the screen a nominal sensitivity of 3.5% as measured as the percentage of “true positives” selected from the sum of the true positives and the false negatives. However, the hit threshold was selected to ensure that 95% of genes with an effect as large as the effect of siCasp8 would be selected as hits. Since Caspase-8 was the gene that had the largest effect in Aza-blanc *et al* screen, other genes in this screen would be expected to have less effect and therefore a reduced probability of being selected as a hit. Further, many of the hits were not rigorously confirmed, with many of them only being targeted by one siRNA. Indeed, the only gene included in the overlap which was confirmed by multiple siRNAs was GSK3 α . Nevertheless, as a group, the genes presented in Table 4-4 have a median score close to zero suggesting the results of this screen do not replicate the screen of Aza-blanc *et al*. siRNA against three of these genes

(ABL2, PRKCQ and PRKRIR) were shown to have an effect on TRAIL-induced cytotoxicity in Figure 3.13. This suggests that the screen presented here is not highly sensitive for the selection of genes previously associated with TRAIL-induced cytotoxicity. While it is concerning that many of the genes previously associated with TRAIL-induced cytotoxicity were not identified, this does not necessarily indicate that the novel genes targeted by siRNAs that did score highly in the screen are not involved in the process. That is, while this data shows that the screen might not be sensitive, it does not offer any data as to the accuracy of the screen.

<i>Gene</i>	<i>Survival</i>		<i>Score</i>		<i>Rank</i>	
	<i>siRNA 1</i>	<i>siRNA 2</i>	<i>siRNA A</i>	<i>siRNA B</i>	<i>siRNA 1</i>	<i>siRNA 2</i>
ABL2	45%	6%	1.22	-0.90	135	1412
BLK	2%	7%	-1.20	-0.95	1455	1655
GSK3 α	20%	42%	0.1	1.52	594	81
GSK3 β	16%	16%	-0.48	-0.10	1050	758
GUK1	19%	24%	0.12	0.48	578	376
IRAK1	19%	17%	0.16	-0.64	545	1188
MAPK1	16%	11%	0.95	0.77	765	929
MAPK10	22%	18%	0.05	0.05	625	630
PRKAA2	22%	17%	0.26	0.10	480	593
PRKCD	8%	3%	-1.06	-1.20	1532	1648
PRKCQ	11%	1%	-1.08	-1.24	1544	1688
PRKRIR	29%	30%	0.46	1.31	390	116
RAF1	29%	32%	0.10	1.04	593	186
ROS1	25%	38%	0.16	1.70	546	65
<i>Median</i>	19%	17%	0.11	0.08	594	694

Table 4-4 Results from this screen of genes previously associated with the TRAIL apoptosis pathway

4.5 Confirmation of hits

While the examination of the scores of siRNAs targeting genes previously associated with TRAIL-induced cytotoxicity showed the sensitivity of the screen may be low, the high scores of the positive controls suggests that the screen can identify siRNAs that do have an effect on the sensitivity of cells to TRAIL. There are several reasons not to take the results of the initial screen at face value. Firstly, while in order for a siRNA to have scored highly in the screen its effect must have repeated in both replicates, the results do not allow for a statistical assessment of the significance and reproducibility of the results. Further, effects elicited by an siRNA maybe due to knock-down of the target gene – implicating this gene in the TRAIL pathway, or effects may also be due to off target effects. In order to address these issues it is necessary to undertake a rigorous confirmation of hits before it is possible to declare that the genes targeted are involved in the TRAIL pathway with any confidence.

There are two possible methods by which genes could be selected for follow-up. Genes targeted by 2 siRNAs which scored greater than 1.6 (Table 4-2), and genes targeted by the top 10 highest scoring siRNAs were selected for follow up (Table 4-3). Thus 14 genes were selected for rigorous confirmation. In order for a gene to be declared as confirmed, transfection of cells with at least two different siRNAs targeting the gene must significantly reduce the sensitivity of the cells to TRAIL-induced cytotoxicity. In order to rule out off-target effects siRNAs that significantly reduce the sensitivity of transfected cells to TRAIL-induced cytotoxicity must also reduce the mRNA level of targeted genes more efficiently than siRNAs that do not significantly reduce sensitivity.

In order to confirm the involvement of hit genes in the TRAIL-induced cytotoxicity pathway, multiple siRNAs targeting the selected genes were tested for their ability to alter sensitivity to the TRAIL ligand. Identical siRNAs to those used in the screen were resynthesised and used where both siRNAs targeting a gene scored over 1.6 (Figure 4.7a, first five genes) otherwise for each gene the siRNA which scored over 1.6 was resynthesised and used and in addition 2 novel siRNAs were used (Figure 4.7a, genes 6-14). In total 11 of the 37 siRNAs tested significantly increased the survival of transfected cells compared to negative control transfected cells on the same plate (using a Student's t-test on log transformed data with a 5% significance level). A total of 4 genes were targeted by two siRNAs that significantly increased the survival of cells treated with TRAIL ligand. The low confirmation rate may, in part, be attributable to the large amount of variation seen in the survival of cells transfected with negative control siRNA on one of the plates in this experiment (Figure 4.7a, siNeg (pl 1)).

siRNAs may fail to affect the sensitivity of transfected cells to TRAIL-induced cytotoxicity due to their inefficiency in reducing the mRNA level of the targeted gene or the lack of involvement of the gene targeted in the TRAIL-induced apoptosis pathway. To distinguish these two possibilities, the ability of the siRNAs used to reduce the mRNA levels of the targeted genes was measured using qRT-PCR (Figure 4.7b). Primers were designed to amplify from mRNA of targeted genes only, and tested for specificity and efficiency. Primers were successfully designed to amplify from 10 of the 14 targeted genes (see Appendix B). As a positive control, the ability of a well characterised siRNA targeting the gene Lamin A/C was also measured. Transfection of the siRNA targeting Lamin A/C reduced the mRNA level to 24% of the negative control level, demonstrating the effectiveness of the transfection and qRT-PCR process. 12 of the 26 siRNAs measured reduced the levels of the target siRNA by more than 70% (Figure 4.7b, dashed line).

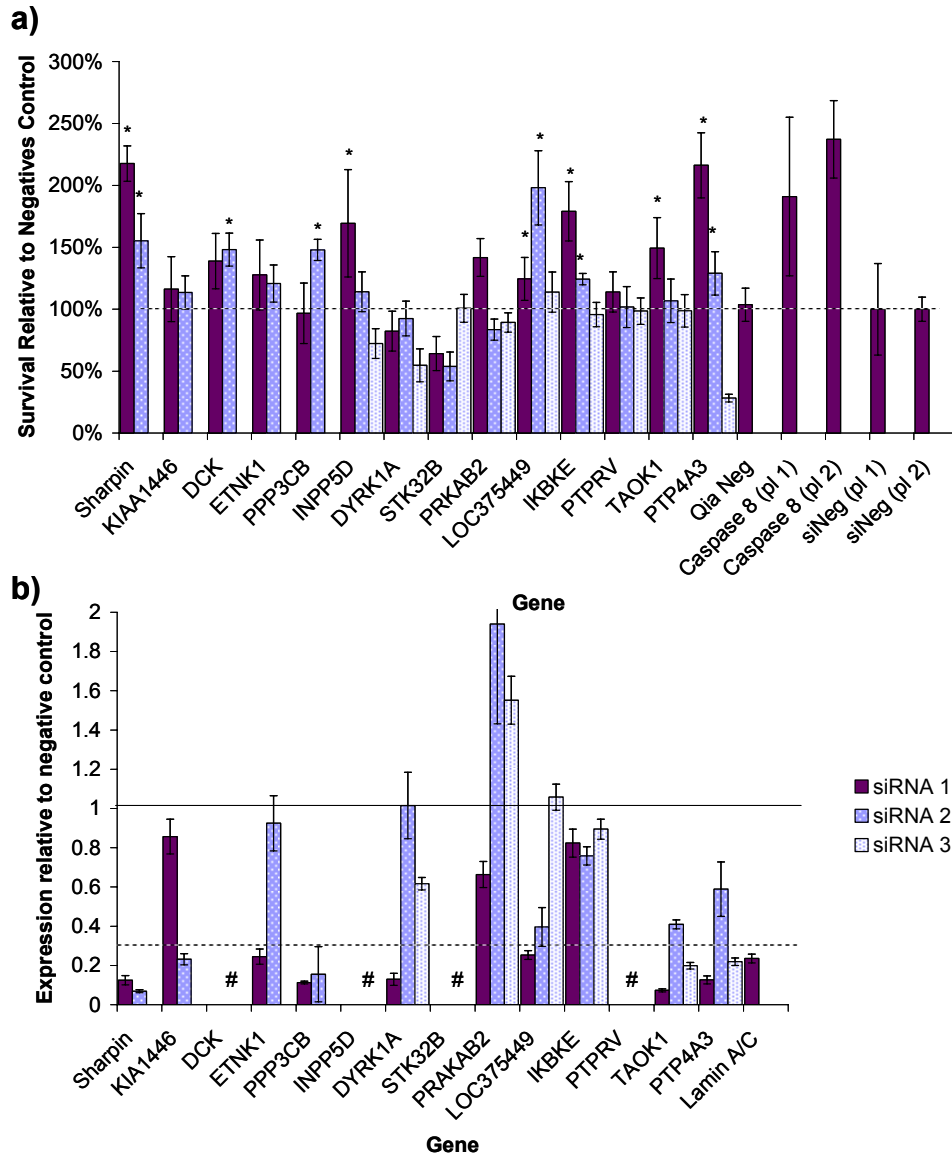


Figure 4.7 Confirmation of the effect of 14 genes from a screen of Kinases and Phosphatases.

a) Effect of siRNAs targeting 'hit' genes on sensitivity to TRAIL induced cytotoxicity. Cells were transfected with either two or three siRNAs targeting genes selected from the kinase and phosphatase screen. After 48 hours viability was measured using alamarBlue and cells were treated with 0.5µg/ml TRAIL. Viability was reassessed 24 hours later. Tests were carried out on two independent plates. Caspase 8 (pl 1) and Caspase 8 (pl 2) – positive controls from plates 1 and 2. siNeg (pl 1) and siNeg (pl 2) – negatives controls from plates 1 and 2. QiaNeg – Qiagen negative control found on screening plates. Data is shown as survival relative to negative control. Dashed line represents survival level of negative control. Error bars represent 1 standard deviation. n = 4. * = result significantly different from negative control using a Student's t-test on log transformed data ($\alpha = 0.05$). b) Effect of siRNAs targeting 'hit' genes on mRNA levels of targeted genes. RNA was isolated from cells transfected with siRNAs targeting genes selected from the kinase and phosphatase screen or Lamin A/C as a positive control. cDNA was prepared by reverse transcription. SYBR green qPCR was carried out in triplicate using primers designed to amplify from mRNA of genes targeted, GAPDH and ACTB. Primers were designed and tested as described in Methods. Primers were successfully designed for 10 of the 14 genes tested. Expression levels are shown relative to negative control and were calculated using a variation of the Pfaffl method to allow normalization to multiple housekeeping genes using GAPDH and ACTB to normalize samples (Hellemans et al. 2007). # = genes for which no primers were successfully designed. Solid line represents 100% of negative control levels and dashed line represents 30% of control levels). Error bars represent 1 standard error of the mean.

<i>Gene</i>	<i>siRNA</i>	<i>TRAIL sensitivity</i>	<i>TRAIL Rank</i>	<i>> 70% KD</i>	<i>KD Rank</i>	<i>Conclusion</i>
Sharpin	1	+	1	+	2	Confirmed Hit
	2	+	2	+	1	
KIAA1446	1	-	1	-	2	Unrepeatable
	2	-	2	-	1	
DCK	1	-	2	N/A	N/A	Unconfirmed
	2	+	1	N/A	N/A	
ETNK1	1	-	2	-	1	Unrepeatable
	2	-	1	-	2	
PPP3CB	1	-	2	+	1	Off-Target
	2	+	1	+	2	
INPP5D	1	+	1	N/A	N/A	Unconfirmed
	2	-	2	N/A	N/A	
	3	-	3	N/A	N/A	
DYRK1A	1	-	2	+	1	Unrepeatable
	2	-	1	-	2	
	3	-	3	-	3	
STK32B	1	-	2	N/A	N/A	Unrepeatable
	2	-	1	N/A	N/A	
	3	-	3	N/A	N/A	
PRKAB2	1	+	1	-	1	Unconfirmed
	2	-	3	-	2	
	3	-	2	-	3	
IKBKE	1	+	1	-	2	Confirmed Hit
	2	+	2	-	1	
	3	-	3	-	3	
LOC375449	1	+	2	+	1	Confirmed Hit
	2	+	1	-	2	
	3	-	3	-	3	
PTPRV	1	-	1	N/A	N/A	Unrepeatable
	2	-	2	N/A	N/A	
	3	-	3	N/A	N/A	
TAOK1	1	+	1	+	1	Unconfirmed
	2	-	2	-	3	
	3	-	3	+	2	
PTP4A3	1	+	1	+	1	Off-Target
	2	+	2	-	3	
	3	-	3	+	2	

Table 4-5 Categorisation of genes selected from confirmation from kinase and phosphatase screen. See text for definition of categories. N/A – not available. KD - Knockdown

Based on these results the genes selected for follow up were categorised into one of four categories by combining the effect of siRNAs on sensitivity to TRAIL-induced

cytotoxicity and the ability of the same siRNAs to reduce the mRNA levels of the targeted genes. Phenotypically active siRNAs are defined as siRNAs, transfection of which leads to a statistically significant reduction in TRAIL-induced cytotoxicity and the efficiency of an siRNA is defined as the degree to which it reduces the level of the intended target transcript.

- **Confirmed Hits** are genes targeted by at least two phenotypically active siRNAs. These two phenotypically active siRNAs must be more efficient than any siRNA tested that was not shown to be phenotypically active. That is, the inactivity of the non-phenotypically active siRNA can be explained by the lack of efficiency.
- **Unconfirmed Hits** are genes targeted by one phenotypically active siRNA. This phenotypically active siRNA must be more efficient than the siRNAs targeting the same gene which were not shown to be phenotypically active. This category also includes genes where the efficiency of siRNAs targeting it are not known.
- **Off-Targets** are genes targeted by both phenotypically active and non-phenotypically active siRNAs. At least one non-phenotypically active siRNAs must be more efficient than at least one phenotypically active siRNA. That is the activity of the phenotypically active siRNAs is at least in part due to off-target effects.
- **Unrepeatable** genes are genes where none of the siRNAs targeting the gene are phenotypically active. That is the original screen result is unrepeatable.

The categorisations of the genes selected for confirmation from the screen are shown in Table 4-5. Three genes are classed as Confirmed Hits (Sharpin, LOC375449 and IKBKE) and a further four genes are classed Unconfirmed Hits (DCK, INPP5D, PRKAB2 and TAOK1). Two genes are classed as confirmed Off-Targets (PPP3CB and PTP4A3). The remaining five genes were not targeted by any siRNAs that significantly altered the sensitivity of cells to TRAIL-induced cytotoxicity and so are classed as unrepeatable.

Of the top ten siRNAs selected for follow up from the ranked list of siRNAs, seven significantly reduced the sensitivity to TRAIL-induced cytotoxicity when retested (Figure 4.7a and Table 4-5), suggesting that the accuracy of this method for selecting 'hit' siRNAs is approximately 70%. However, only three of the genes targeted by these siRNAs were confirmed as hits by being targeted by two independent siRNAs that, when transfected, caused a significant reduction in sensitivity to TRAIL-induced cytotoxicity, and which reduce the level of mRNA more efficiently than siRNA which do not have an effect on TRAIL sensitivity. This suggests that the accuracy of this method for selecting 'hit' genes is 30%. This could possibly be raised to 60% if all the unconfirmed genes were confirmed. Of the five genes selected for being targeted by two siRNAs scoring greater than 1.6 in the screen,

only one gene confirmed, suggesting that the accuracy of this method for selecting 'hit' genes is approximately 20%, rising to 30% if the unconfirmed genes were also confirmed.

The screen has clearly not been as effective as might have been expected given the optimisation experiments, particularly with regard to finding genes previously associated with the pathway. It should be remembered however, that many of the previously associated genes are from the Aza-blanc screens. These hits were not confirmed with the appropriate rigor, and so it impossible to tell how many are genuinely involved in the pathway. The number of genes selected from the screen that confirmed is also lower than might have been expected. This may in part be due to the rigorous confirmation process applied. While the number of confirmed hits is small, the confidence that these hits are genuinely involved in the process is high

4.6 Experimental characterisation of confirmed hits

From rigorous follow up of the screen of siRNAs targeting 897 kinases, phosphatases and associated genes, three genes passed the confirmation process. These genes are targeted by two siRNAs that significantly reduce the sensitivity to cells to TRAIL-induced cytotoxicity and reduce the mRNA level of the target gene to a greater extent than any siRNA tested that does not significantly reduce the sensitivity of cells to TRAIL-induced cytotoxicity. It should be noted that in one case (IKBKE), although the phenotypically active siRNAs are more efficient than the non-active siRNAs, the difference in efficiency is small, while the difference in sensitivity is large. However, since the gene is targeted by two siRNAs that are phenotypically active and given that IKBKE is an activator of NF- κ B which has been implicated in control of TRAIL sensitivity previously (1.3.2.6) it was decided that it was worth investigating this gene further. To investigate the significance of these genes to the TRAIL-induced apoptosis pathway a series of experiments were undertaken to characterise these them.

4.6.1 Effect of knock-down of confirmed hits on TRAIL ligand dependent activation of caspases.

As noted in earlier chapters, the assay implemented in the screening for and confirmation of, genes involved in the TRAIL pathway has measured the effect of siRNAs on TRAIL-induced cytotoxicity rather than TRAIL-induced apoptosis. In order to establish that hit genes affect TRAIL-induced apoptosis, the effect of siRNA mediated knock-down of hit genes on the TRAIL-ligand dependent activation of several caspases was investigated. As well as confirming the involvement of these genes in TRAIL-induced apoptosis, in

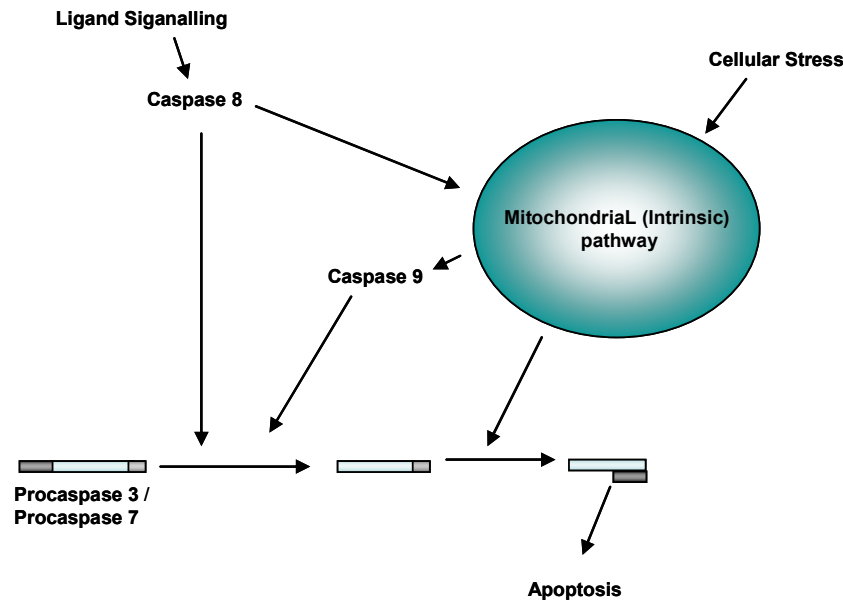


Figure 4.8 Order of caspases in the caspase cascade

In addition to TRAIL-induced cytotoxicity, these experiments will indicate the point in the apoptotic pathway at which the genes act. Most genes involved in TRAIL-induced apoptosis would be expected to affect the activation of the executioner caspases, Caspase-3 and Caspase-7 by the TRAIL ligand. Genes involved in the regulation of the intrinsic, mitochondrial pathway, or the connection between the extrinsic and intrinsic pathways would be expected to affect the induction of Caspase-9 as well as caspases -3 and -7. Genes involved in the regulation of the extrinsic pathway would be expected to affect Caspase-8 in addition to caspases -9,-3 and -7 (Figure 4.8). The effect of the knock-down of hit genes on the induction of these caspases was determined using luminescent caspase assays to compare caspase activity in TRAIL treated vs. mock treated cells (Figure 4.9).

As expected TRAIL treatment of cells increases the activity of Caspase-8 more than three fold (from 1,026 to 3,418, Figure 4.9a). The levels of Caspase-8 activity in TRAIL treated, siCasp8 transfected cells are 45% of those in negative control transfected cells (Figure 4.9a). Reduction of Caspase-8 levels in TRAIL-treated cells is significant in cells transfected with both siRNAs against IKBKE (siIKBKE.1 and siIKBKE.2) and one of the two siRNAs against each of Sharpin and LOC37449. Levels are reduced to 60% and 73% of the negative control respectively for cells transfected with siIKBKE.1 and siIKBKE.2 and to 36% and 38% for cells transfected with siRNA 1 targeting Sharpin (siSharpin.1) and siRNA 2 targeting LOC375449 (siLOC375449.2) respectively. Transfection of siRNA 2 against Sharpin (siSharpin.2) and siRNA 1 against LOC375449 (siLOC375449.1) did not significantly reduce the activity of Caspase 8 in TRAIL treated cells compared to the negative control.

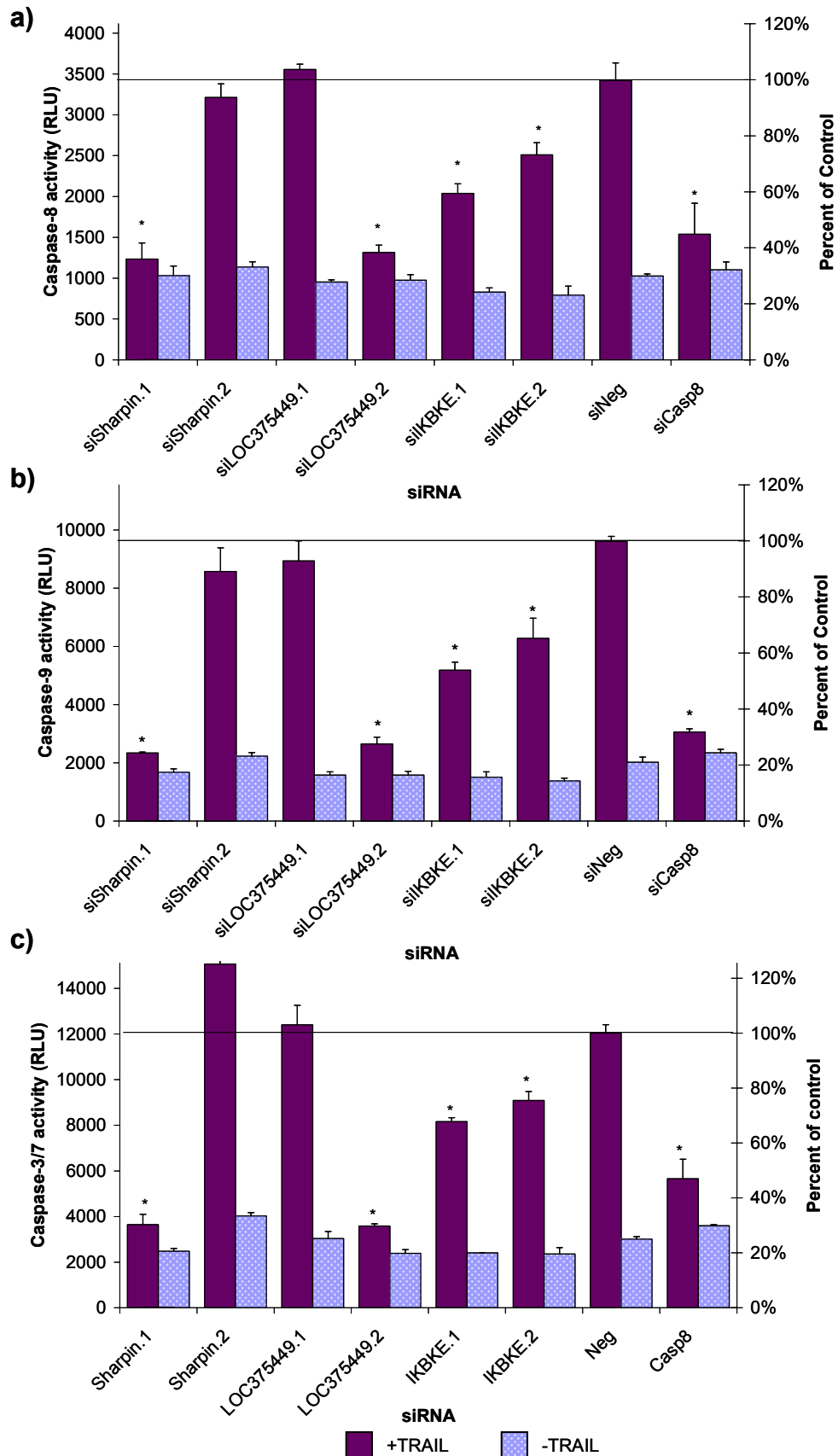


Figure 4.9 Effect of siRNAs targeting confirmed hits on TRAIL-induced caspase activation

Cells were transfected with siRNAs targeting confirmed hits. 48 hours later cells were treated with either 0.5µg/ml TRAIL or media for six hours. Caspase-8 (a), Caspase-9 (b) or Caspase-3/7 (c) luminescent assay reagent was added to cells and incubated for 1 hour before luminescence was determined. Error bars represent 1 standard deviation. n = 3. * = significantly different from negative control using a Student's t-test (Bonferroni corrected $\alpha = 0.05$). Horizontal line represents negative control level. RLU = relative luminescent units

These results are almost identical to the results obtained by measuring the effect of transfecting these siRNAs on the activity of Caspase-9 (Figure 4.9b) and Caspases 3/7 (Figure 4.9c). The correlation coefficient between the effects on Caspase-8 and the effects on Caspase-9 is 0.99 ($r^2 = 0.98$) and the correlation coefficient between effects on Caspase-8 and Caspases 3/7 is 0.95 ($r^2 = 0.90$). The effect of knockdown on the level of Caspase-9 activity is generally stronger: transfection of siCasp8 reduces the activity of Caspase-9 in TRAIL treated cells to 31% of activity in negative control transfected cells, compared to reducing the activity of Caspase-8 itself to 45% of control levels.

It is unclear why only one siRNA targeting Sharpin and LOC375449 significantly reduce the levels of caspase activity (Figure 4.9), when both siRNAs targeting both these genes reduce the level of cytotoxicity induced by TRAIL treatment (Figure 4.7). However, those siRNAs which do not significantly reduce the levels of caspase activity are the siRNAs which reduced the TRAIL-induced cytotoxicity least of the two siRNAs targeting each gene. There is a strong correlation between the effect of siRNAs targeting hit genes on TRAIL-induced cytotoxicity and their effects on the level of Caspase-8 activity ($r^2 = 0.80$).

It can be concluded that there is strong evidence that the genes identified as confirmed hits from the screen of kinases, phosphatases and associated genes affect TRAIL-induced apoptosis as well as TRAIL-induced cytotoxicity. Further there is evidence that all three genes act to regulate the pathway at or above the level of Caspase-8 activation.

4.6.2 Effect of knock-down of confirmed hits on the sensitivity of HeLa cells to a selection of apoptosis inducing conditions

Genes regulating the sensitivity of cells to TRAIL-induced apoptosis may do so at several levels. They may be specifically involved in the regulation of the TRAIL-induced apoptosis pathway. In this case knock-down of these genes should affect the sensitivity of cells to TRAIL-induced apoptosis, but not the sensitivity of cells to apoptosis induced by other conditions. Alternatively genes may be involved in regulating the sensitivity of cells to ligand-induced apoptosis. In this case knock-down of these genes should affect the sensitivity of cells to both TRAIL-induced apoptosis and also the sensitivity of cells to other apoptosis inducing ligands, such as FAS ligand. Finally genes may be involved in the regulation of the general sensitivity of cells to apoptosis. In this case knock-down of the genes should affect the sensitivity of cells to TRAIL-induced apoptosis, apoptosis induced by other ligands, and sensitivity to apoptosis induced by non-ligand apoptosis inducers such as hydrogen peroxide or UV radiation.

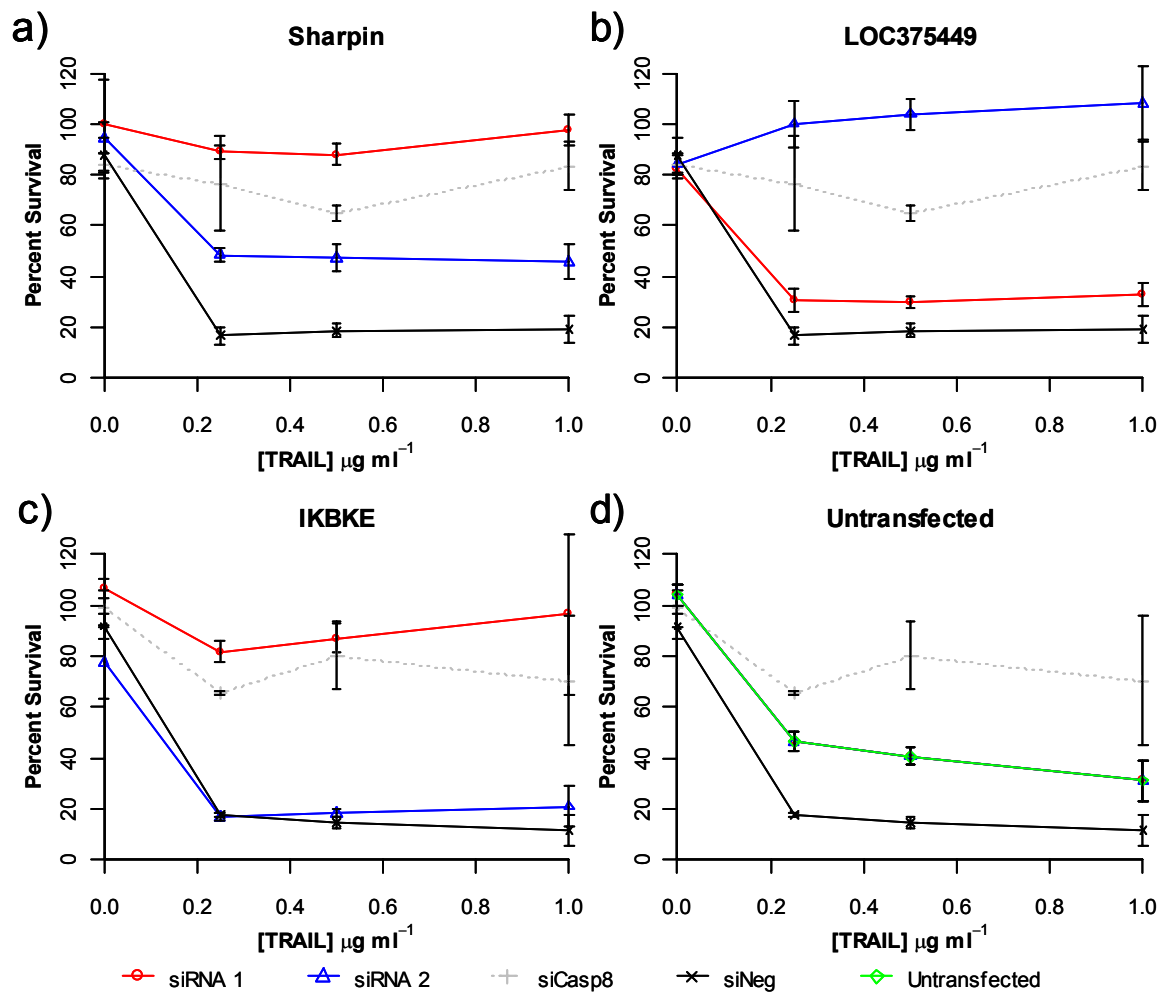


Figure 4.10 Knockdown of confirmed hit genes affects sensitivity of cells to a range of concentrations of TRAIL ligand.

Cells were transfected with siRNAs targeting a) Sharpin, b) LOC375449, c) IKBKE, siCasp8 or siNEG or d) mock transfected. Viability was measured using alamarBlue 48 hours later. Cells were treated with either 0.25 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$ TRAIL ligand or serum free media for 24 hours and the viability reassessed. Error bars represent 1 standard deviation, $n = 3$.

In order to further confirm the effect of hit siRNAs on TRAIL-induced cytotoxicity, and also to provide a comparison for the effect of other apoptosis inducers, the effects of knockdown of confirmed hit genes on the sensitivity of cells to TRAIL-induced apoptosis at a range of TRAIL concentrations was determined (Figure 4.10). The effects of the transfection of siRNAs targeting Sharpin and LOC375449 reflect the effects seen in the confirmation experiments. siSharpin.1 and siSharpin.2 increased the number of cells which survived treatment with 1 $\mu\text{g/ml}$ TRAIL from 19% to 98% and 46% respectively, while 83% of cells transfected with siCasp8 survived (Figure 4.10a). Similarly transfection with siLOC375449.1 and siLOC735449.2 increased survival of cells treated with 1 $\mu\text{g/ml}$ TRAIL from 19% to 33% and 106% respectively (Figure 4.10b). The concentration of TRAIL had little effect on survival in both cases. Knock-down of IKBKE by siIKBKE.1 increased the survival of cells treated with 1 $\mu\text{g/ml}$ TRAIL from 11% to 97% compared with siCasp8,

which increased survival to 71%. The concentration of TRAIL had little effect on the level of cytotoxicity induced by TRAIL. However, knockdown IKBKE by siIKBKE.2 only increased survival of cells treated with 0.5µg/ml TRAIL from 14% to 18%, although the increase was marginally more substantial when cells were treated with 1µg/ml where siIKBKE.2 increased survival from 11% to 21% (Figure 4.10c). While at 7% the standard deviation of the mean survival of siIKBKE.2 transfected cells treated with 1µg/ml TRAIL is within the range seen for other siRNAs in this experiment, the standard deviation as a proportion of the mean, that is the coefficient of variance, is much higher for siIKBKE.2 than for data from other siRNAs. This pattern is in line with previous findings with siIKBKE.1 having a larger effect on both TRAIL-induced cytotoxicity and TRAIL-induced caspase activation than siIKBKE.2 (Figure 4.7a and Figure 4.9), but here the pattern is much more pronounced. This presents a difficulty. Transfection of siIKBKE.2 produced a significant change in sensitivity to TRAIL-induced cytotoxicity in confirmation experiments, and also reduced the level of TRAIL-induced caspase activity, yet here, doesn't produce a significant change in TRAIL-induced cytotoxicity, although it does cause a non-significant reduction in sensitivity. Therefore the weight of evidence is in favour of this gene being involved in TRAIL-induced apoptosis. Similar to findings in the assay development experiments, but unlike findings from the screen, untransfected cells are more resistant to TRAIL-induced cytotoxicity than negative control transfected cells.

In order to determine the involvement of confirmed hit genes in regulation of apoptosis induced by other ligands, the effect of knockdown of these genes on the sensitivity of cells to FAS ligand-induced apoptosis was determined. (Figure 4.11). An average of 19% of negative control transfected cells survived treatment with 100ng/ml recombinant FAS ligand plusTM (FAS ligand fused to a FLAG epitope, referred to as FAS ligand from here on). An average of 76% of siCasp8 transfected cell survived treatment with 100ng/ml FAS ligand (Figure 4.11). Knockdown of Sharpin by siSharpin.1 but not siSharpin.2 reduces the sensitivity of cells to FAS ligand at all concentrations tested, with 43% of cells surviving treatment with 100ng/ml of FAS ligand, compared with 20% of negative control transfected cells ($p = 0.009$, calculated using Student's t-test on log transformed data) and 26% of siSharpin.2 transfected cells (Figure 4.11a). Knockdown of LOC375449 by both siRNAs targeting this gene reduced the sensitivity to cells to FAS ligand at all concentrations tested. Transfection with siRNAs siLOC375449.1 and siLOC375449.2 increased survival of cells treated with 100ng/ml of FAS ligand to 40% and 57% respectively compared with 20% of negative control transfected cells ($p = 0.011$ and 0.004 respectively, Figure 4.11b). As with

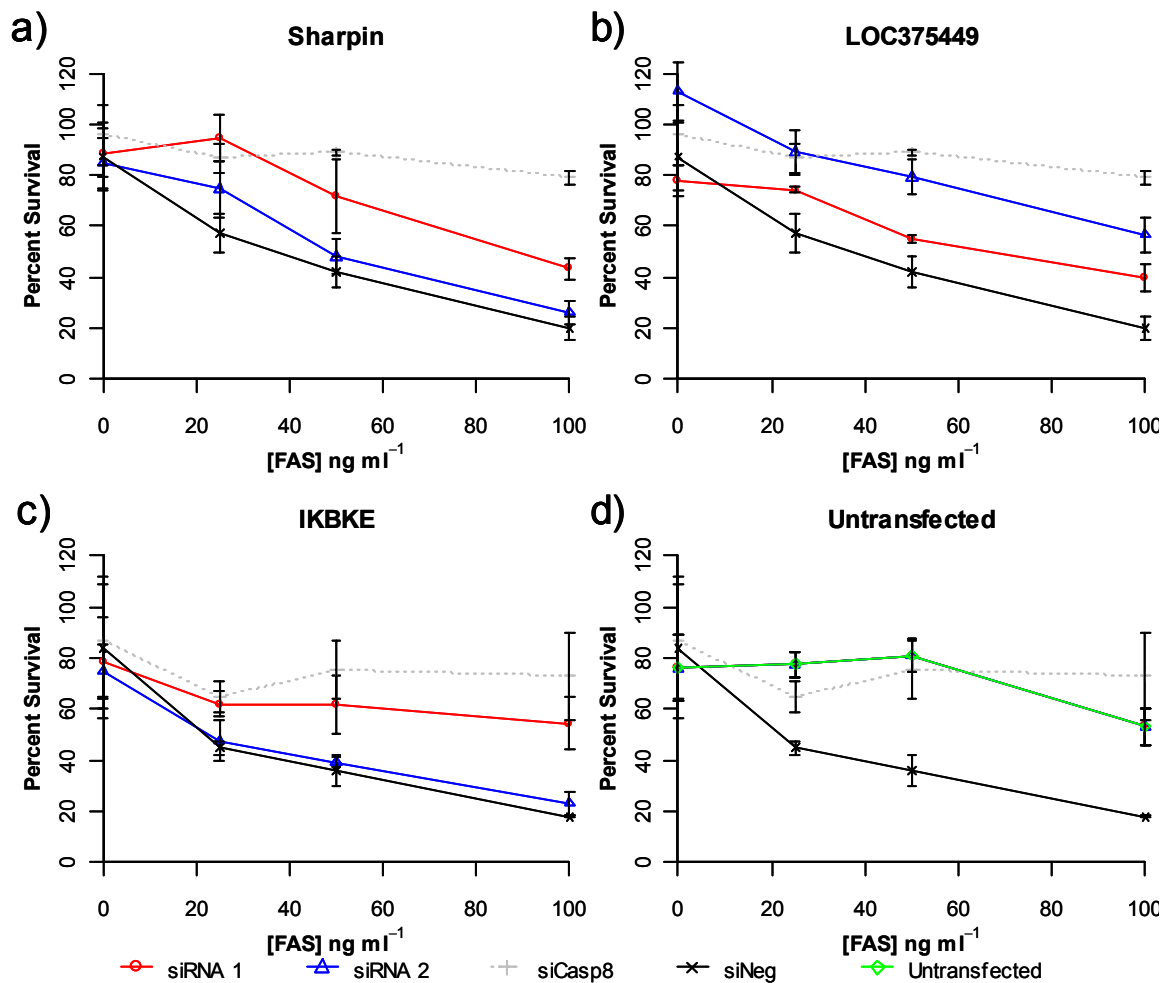


Figure 4.11 Knockdown of confirmed hit genes affects sensitivity of cells to FAS ligand induced apoptosis.

Cells were transfected with siRNAs targeting a) Sharpin, b) LOC375449, c) IKBKE, siCasp8 or siNEG or d) mock transfected. After 48 hours viability was assessed using alamarBlue. Cells were then treated with the concentration of FAS ligand Plus™ indicated in serum free media or serum free media only for 24 hours and viability was reassessed. Error bars represent 1 standard deviation, n = 3

the effect of knock-down of IKBKE on TRAIL sensitivity, knockdown of IKBKE by siIKBKE.1, but not siIKBKE.2 reduced sensitivity of cells to FAS ligand induced apoptosis. As with sensitivity to TRAIL-induced cytotoxicity, the transfection processes itself increases the sensitivity of cells to FAS (Figure 4.11d).

Knockdown of all three confirmed hit genes affects the sensitivity of cells to apoptosis inducing ligands other than TRAIL, although the effects are not as strong. siRNAs that have a small effect on the sensitivity of cells to TRAIL-induced apoptosis, do not have a significant effect on FAS ligand induced death. This is similar to the effect of increased cFLIP levels. Over-expression of cFLIP has a stronger inhibitory effect on TRAIL-induced apoptosis than it does on FAS-induced apoptosis (Irmeler et al. 1997).

The role of the confirmed hit genes in the regulation of cell death induced by physiological conditions was examined by determining the sensitivity of cells to H₂O₂-(which

increases oxidative stress) and UV radiation- (which leads to DNA damage) induced cell death (Figure 4.12 and Figure 4.13). High concentrations of H₂O₂ killed all cells, irrespective of siRNA transfection. At lower concentrations significantly more siNeg transfected cells died than siCasp3 transfected cells (average survival after treatment with 100µM H₂O₂ was 36% and 86% respectively). Knock-down of Sharpin had no effect at either 200µM or 100µM H₂O₂, however knockdown of Sharpin (by siSharpin.1 only) did significantly increase the survival of cells treated with 50µM H₂O₂, from 81% to 122%, with a p-value of 0.010 (Figure 4.12a). Knock-down of LOC375449 had an effect on sensitivity of cells only at 100µM H₂O₂, however the effect of neither siRNA is significant at the 5% level (survival of 70% and 73% for siLOC375449.1 and siLOC375449.2 transfected cells compared with 43% for siNeg transfected cells, p-value = 0.72 and 0.62 respectively, Figure 4.12b). siIKBKE.2 had no effect on the sensitivity of cells to H₂O₂. However siIKBKE.1 had a large, significant effect, increasing the survival of cells treated with 100µM from 30% to 77% (Figure 4.12c, p value = 0.003).

It is known that high enough concentrations of H₂O₂ induce a necrotic cell death rather than an apoptotic cell death. This could explain the lack of effect of knock down of IKBKE and Caspase-3 at higher concentrations, when a large effect is observed at lower concentrations (Nosseri, Coppola & Ghibelli 1994).

Of all six siRNAs, only siIKBKE.1 has a large and significant effect on the sensitivity of cells to UV radiation-induced cell death (Figure 4.13c). siIKBKE transfection increased the survival of cells treated with 100 J/ml² UV radiation from 31% to 85% (p-value = 0.0002). siSharpin.1 had a smaller effect on the sensitivity of cells to UV radiation, increasing the survival of cells exposed to 100 J/ml² UV radiation from 28% to 50%, (p-value = 0.02, Figure 4.13a). It is worth noting that the positive control siCasp3 had a similar effect, increasing survival from 28% to 51%. Unfortunately the interpretation of data on the sensitivities of cells to UV radiation is hampered by low viability in cells supposedly not exposed to UV radiation, particularly the negative control. One possible explanation for this is that a defect in the experimental protocol allowed the cells which were not to be exposed to UV to be exposed, through insufficient shielding. This would account for the observed increased survival of supposedly unexposed cells transfected with siRNAs which increase the survival of cells exposed to UV radiation. That is, if they were accidentally exposed to some level of UV radiation, the transfection of an siRNA regulating apoptotic responses would protect them, while transfection of the non-targeting siNeg would not.

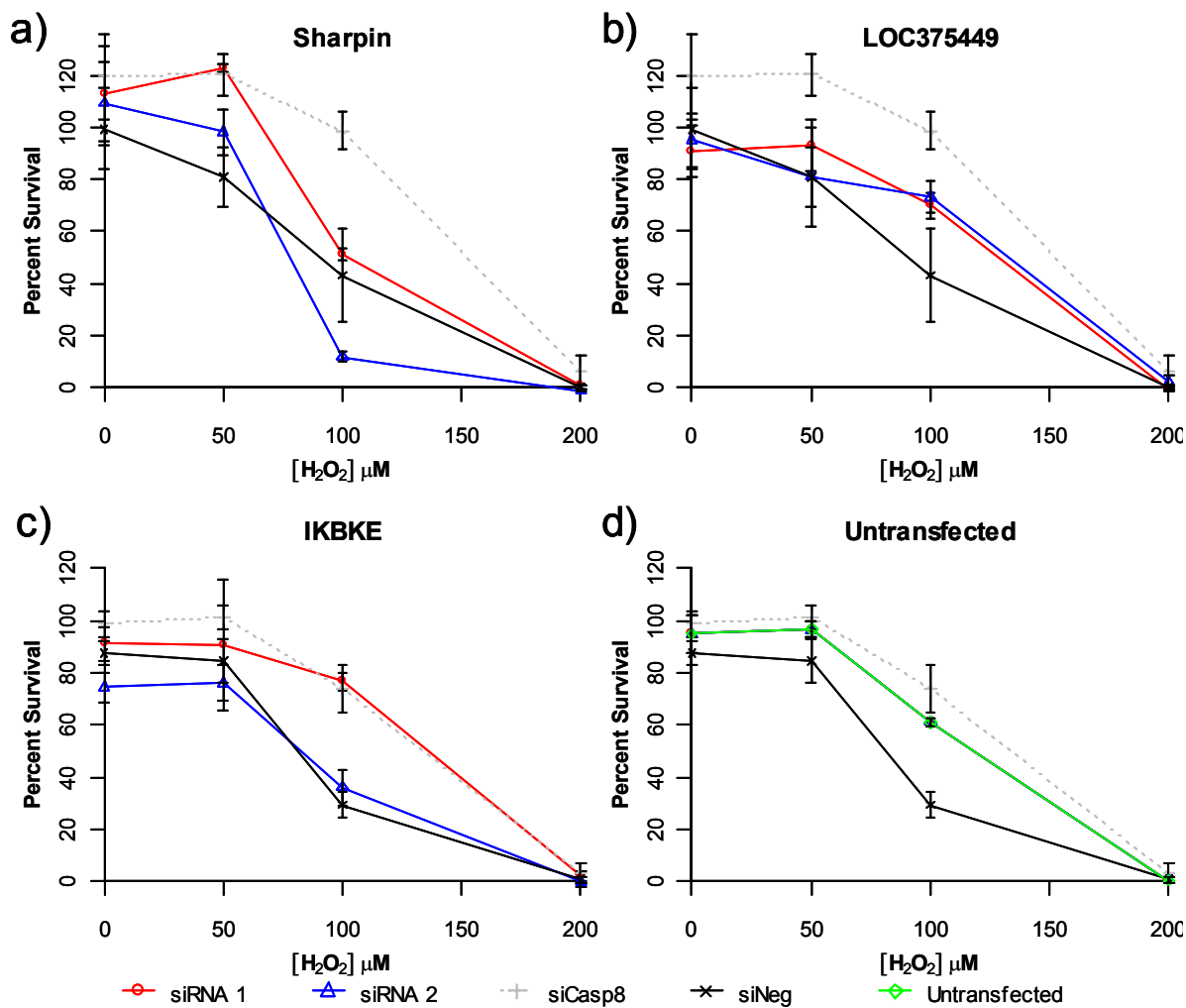


Figure 4.12 Effect of knockdown of confirmed hit genes on sensitivity to H_2O_2 induced cytotoxicity.

Cells were transfected with siRNA targeting a) Sharpin, b) LOC375449, c) IKBKE, an siRNA targeting Caspase-3 (siCasp3) as a positive control or siNeg or d) mock transfected. After 24 hours the viability of cells was measured using alamarBlue. Cells were then incubated in serum free media containing the concentration of H_2O_2 indicated for 24 hours and viability was reassessed. Error bars represent 1 standard deviation, n = 3.

The results of the biological investigations undertaken are summarised in Table 4-6. The strength of the effects of transfection with each siRNA in each of the assays correlated with strength of the effects in the original confirmation experiment (Figure 4.7a). All of the siRNAs that significantly reduced the activity of Caspases -3 and -7 in TRAIL treated cells, also significantly reduced the activity of Caspases -8 and -9, suggesting these genes regulate the apoptotic pathway at the level of Caspase-8 activation. Consistent with this knockdown of LOC37449 reduces the sensitivity of cells to FAS ligand induced cell death as well as TRAIL-induced cell death, but not to H_2O_2 induced or UV induced cell death. Knockdown Sharpin also protects against both TRAIL and FAS ligand induced cell death. It may also protect to some extent against H_2O_2 induced cell death and UV induced cell death, but this effect is much weaker. In contrast knockdown of IKBKE by the siRNA that gave the strongest protection against TRAIL-induced cell death also had a strong protective effect

against FAS, H₂O₂ and UV induced cell death.

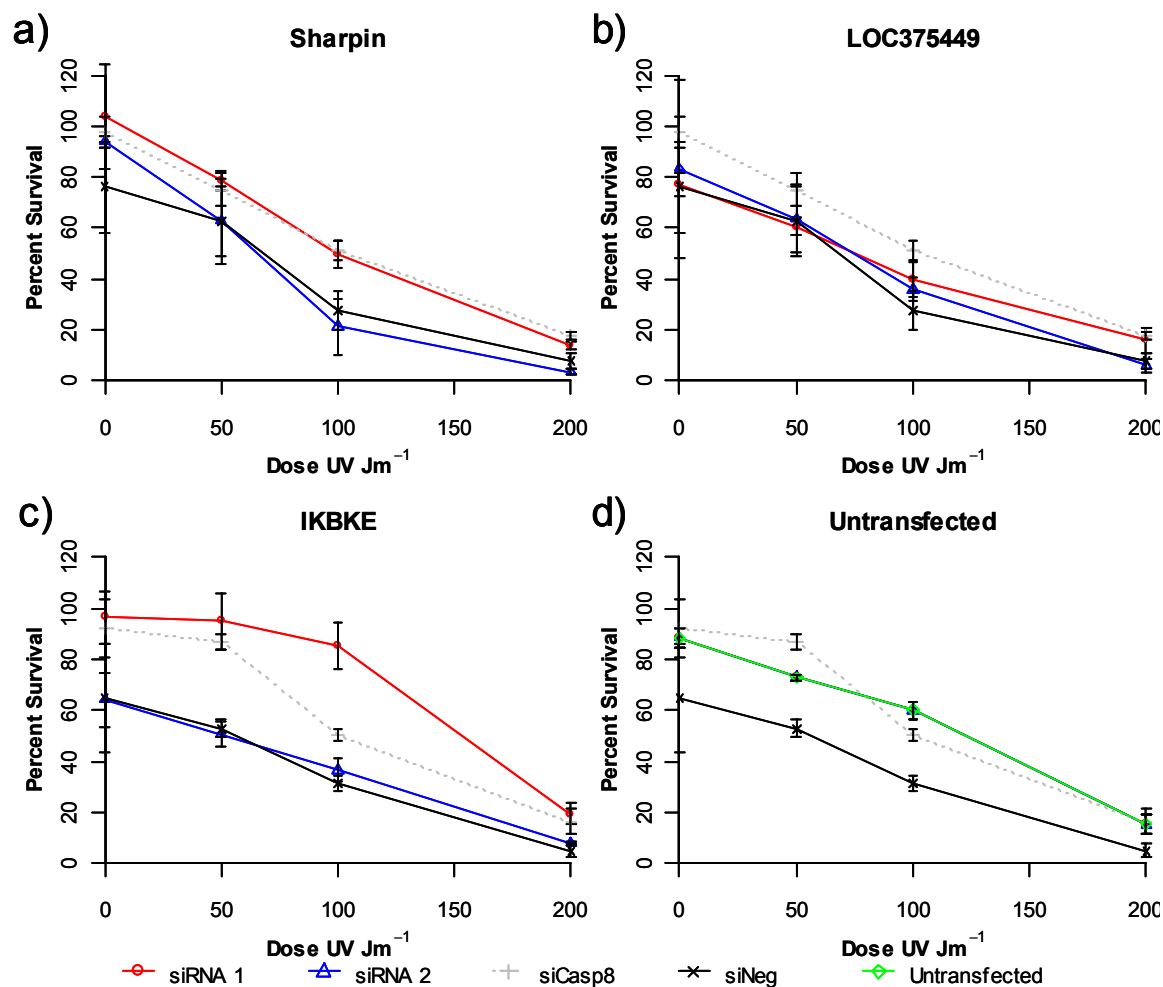


Figure 4.13 Effects of knockdown of confirmed hit genes on sensitivity to UV radiation induced cell death.

Cells were transfected with siRNAs targeting a) Sharpin, b) LOC375449, c) IKBKE, Caspase -3 (siCasp 3), siNEG or d) mock transfected. 48 hours later viability of cells was assessed using alamarBlue. Cells were exposed to doses of UV radiation indicated. Cells were incubated for 24 hours in serum free media and viability was reassessed. Error bars represent 1 standard deviation. n = 3.

siRNA	Caspase Cascade			Apoptosis Inducers			
	Caspase 8	Caspase 9	Caspase 3/7	TRAIL	FAS	H ₂ O ₂	UV
siSharpin.1	+	+	+	+	+	-/+	-/+
siSharpin.2	nsd	nsd	nsd	+	nsd	nsd	nsd
siLOC375449.1	nsd	nsd	nsd	+	+	nsd	nsd
siLOC375449.2	+	+	+	+	+	nsd	nsd
siIKBKE.1	+	+	+	+	+	+	+
siIKBKE.2	+	+	+	nsd	nsd	nsd	nsd

Table 4-6 Summary of experimental characterisation of confirmed hits from screen of Kinase and Phosphatase screen.

Nsd – No significant difference under any of the tested conditions.

4.7 Discussion and conclusions

A screen of genes annotated as kinases, phosphatases or kinase/phosphatase associated was carried out (Figure 4.5 and Figure 4.6). Fourteen genes were selected for confirmation by virtue of being targeted by two siRNAs scoring highly in the screen or being targeted by one siRNA scoring very highly (Table 4-2 and Table 4-3). Of these genes three could be designated confirmed hits, while four were designated as unconfirmed hits, two as off-target hits and five as unreproducible (Table 4-5 and Figure 4.7).

The three confirmed hits were further investigated by experiments to measure the effect of knock-down of these genes on TRAIL ligand induced activation of several caspases (Figure 4.9) and their ability to affect sensitivities to apoptosis inducers other than TRAIL (Figure 4.10, Figure 4.11, Figure 4.12 and Figure 4.13).

4.7.1 The Screen

Different normalization and data transformation strategies were explored. Median normalization produced the most consistent results and was thus selected as the normalization method for data analysis (Figure 4.3). Normalisation methods based on values of control wells are sensitive to the effects of small numbers of outliers in these wells, particularly when only two replicates of each well are present on each plate.

It was observed that there was a strong relationship between mean normalized survival and variance between screen replicates in mean/standard deviation plots of the screen results (Figure 4.4a). This relationship was also observed in scatter-plots of one replicate of the screen against the other and in the spread of scores for siCasp8 transfected wells compared to negative control transfected wells (Figure 4.5d). While such relationships can complicate analysis, log transforming data did not remove this relationship and may have made it stronger (Figure 4.4b).

Three positive controls were included in each plate. These controls had previously been observed to affect the sensitivity of cells to TRAIL to different degrees, with siCasp8 having the strongest effect, followed by siBID and then siSMAC. Transfection of each of these controls reduced the sensitivity of cells to TRAIL-induced cytotoxicity as expected (Figure 4.5d), although there was a reduction in the separation between the positive controls and negative controls compared to the assay development experiments (as measured by Z' -factor). This could represent the extra variability that is inevitable with an increase in throughput, or the reduced concentration of TRAIL used in the screen compared to earlier

experiments (0.25 μ g/ml here compared with 1 μ g/ml previously) despite the fact that previous experiments showed little reduction in cytotoxicity when concentration of TRAIL was reduced.

Comparison of the inter-replicate correlation and the inter-siRNA correlation reveals that there is a much higher degree of reproducibility between replicates of the same siRNA than there is between different siRNAs targeting the same gene (Figure 4.5a and Figure 4.5b). This demonstrates that while the screen could be regarded as accurate at selecting highly active siRNAs, the interpretation of this in terms of selecting hit genes is more difficult. This was also seen in the confirmation process where 70% of siRNAs from the top ten scoring siRNAs in the screen reproducibly reduced the sensitivity of cells to TRAIL-induced cytotoxicity, but only 30% of genes targeted by siRNAs in the top ten scoring siRNAs were confirmed. There are two possible explanations for this. Either the siRNAs not eliciting a phenotype do not sufficiently knock down the target mRNA, or alternatively the siRNAs that are eliciting a phenotype are doing so through the knock-down of an off-target mRNA not targeted by the second siRNA. Two lines of evidence suggest the former explanation applies in more cases than the latter. Firstly, in five cases, when attempts were made to confirm genes targeted by only a single siRNA by obtaining further siRNAs, these siRNAs did not knock-down the mRNA of the targeted gene as efficiently as the siRNA originally selected from the screen. In two of these cases the original siRNA elicited a significant phenotype while the additional, less efficient siRNAs did not. These are two of the four “unconfirmed hits”, with the other two being genes for which siRNA efficiency was not measured. Effects of siRNAs targeting these genes could still turn out to be due to off-target effects. However, this interpretation is also suggested by the finding that only one of the 28 siRNAs targeting genes previously associated with the TRAIL-induced apoptosis pathway scored over the hit threshold score of 1.6. It must, however, be remembered that the threshold was set to capture 95% of siRNAs that had an effect of the same strength as knock-down of Caspase-8. This same cut off would have only selected 70% of wells with siRNAs targeting BID and only 2% of wells with siRNAs target SMAC/DIABLO, even though transfection with this siRNA had a clear (albeit small) effect on the sensitivity of cells to TRAIL-induced cytotoxicity. Further the majority of genes identified in the Aza-Blanc screen were not rigorously confirmed. In the majority of cases only a single siRNA targeting the gene was used, the efficiency of the knockdown elicited was not measured and no statistics were employed to determine if differences were significant and so it is possible that a number of these are false positives (Aza-Blanc et al. 2003).

The low correlation between the effects of an siRNA on TRAIL-induced cytotoxicity and the effect of other siRNAs targeting the same gene would suggest that the score of both siRNAs targeting the gene must be taken into account when selecting genes for follow up. Despite this, selecting genes for follow up on the basis of the top scoring siRNAs proved more efficient than selecting genes for follow up on the basis of two siRNAs scoring higher than a pre-selected threshold based on the distribution of the control siRNA scores, since the former method led to the confirmation of 3 genes from 10 as genuine hits, an accuracy of 30%, while the latter method led to the confirmation of 1 from 5 genes, an accuracy of 20%. It is to be noted that these differences do not reach statistical significance, indeed 30% is similar to 20% given the sample sizes. However, no gene was confirmed which was selected by virtue of being targeted by two siRNAs scoring over 1.6 in the screen that was not also selected by being targeted by one siRNA in the top ten siRNAs. The small sample size also points to another problem with selecting genes in this way – the small number of genes selected. While a higher threshold may have helped to select hit genes, the small number of genes with two siRNAs scoring higher than 1.6 suggests that this strategy would select very few genes.

Assessing the sensitivity and accuracy of the screen is difficult. 70% of siRNAs from the top ten siRNAs reconfirmed, this means that the accuracy of the screen at selecting siRNAs which have an effect is 70%, but only for siRNAs in the top ten, undoubtedly the accuracy will fall further down the ranked list of siRNAs. Further, only 30% of genes targeted by these siRNAs were confirmed (although with 3 genes “unconfirmed” this could rise to 60%), suggesting that the accuracy of the screen for selecting hit genes is much lower, even for this portion of the ranked list of siRNAs. In terms of sensitivity, 95% of siCasp8 transfected wells scored more than 1.6 (by design), but only 70% of siBID transfected wells and 2% siSMAC transfected wells scored more than this threshold. Further, only 3.5% of siRNAs targeting genes previously associated with TRAIL-induced apoptosis scored more than this threshold, although it is possible that some of these are false positives (see above). Genes were also selected for confirmation by virtue of being targeted by an siRNA in the top ten highest scoring siRNAs in the screen. The lowest scoring of these had a score of 3.24. 66% of wells transfected with siCasp8, 16% of wells transfected with siBID and none of the wells transfected with siSMAC scored higher than this threshold. None of the wells transfected with negative controls siRNAs scored higher than this threshold. However, it should be noted that this criteria was not designed to definitively separate all hits from all none hits, but rather to select high confidence candidates for confirmation.

These calculations rely on classifying genes as either hits or non-hits, assuming that the distribution of siRNA scores is a mixture of two distributions: the distribution of siRNAs that do affect TRAIL-induced cytotoxicity and the distribution of those that do not. In a pathway model of cellular signalling, if a gene in the pathway is present then the pathway is intact and signals. In the absence of this component the pathway is incomplete and does not signal. As such, genes are either involved or not in the process. Thus the position of an siRNA score in the distribution of all siRNA scores is purely determined by the efficiency of the knockdown, and the technical variation in the experiment – differences in transfection efficiency, variation in the measurement of the phenotype etc. In a network model of cellular signalling, each component has a quantitative effect on the output signal of the network. Thus there is a relationship between the knock-down efficiency and network output. If network output falls below a certain level then the apoptotic program is not initiated in response to the TRAIL ligand. Crucially however, this relationship is different for different genes. As such, the distribution of siRNA scores depends on two distributions: the variations due to technical variation in the system (transfection efficiency, assay variation, variation in the state of other network components etc.) and also variation due to the different quantitative effects on network output. In this case it is not only difficult to divide genes into two categories: those that have an effect on TRAIL-induced cytotoxicity, and those that do not, it is also somewhat meaningless to do so. The distribution of siRNA scores seen in the screen is continuous over a large portion of the total range of scores (Figure 4.6), suggesting a continuous distribution of effects. However this could reflect the distribution of siRNA mediated knock-down efficiencies and technical variation and disentangling the contribution of these different sources of variation in siRNA score is difficult given only two siRNAs targeting each gene and only two replicates for each siRNA.

The failure to identify genes previously associated with TRAIL-induced apoptosis, along with the small number of genes for which the two siRNAs calls for a critical evaluation of the design of the screen. In particular, the decision only to include two replicates of the screen, leads to the necessity of using the conservative minimum replicate summary. It would have been useful to have repeated the third replicate of the screen and then compared the results from a mean of three replicate design to those from a minimum of two replicates design.

Although previously identified genes were not identified in the screen presented here, the screen has been successful in identifying siRNAs that have a large and reproducible affect the sensitivity of cell to TRAIL-induced cytotoxicity with a low false positive rate. The

involvement of three novel genes targeted by these siRNAs was confirmed. As such the screening system presented here has proved useful for identifying new genes involved in the TRAIL-induced apoptosis pathway.

4.7.2 The Hits

Three confirmed hits were identified from the screen. Each of these genes is targeted by two siRNAs that significantly reduced the sensitivity of the transfected cells to TRAIL-induced cytotoxicity and also reduced the target mRNA more efficiently than siRNAs targeting the same gene that did not significantly reduce the sensitivity of transfected cells to TRAIL-induced cytotoxicity (Figure 4.7 and Table 4-5).

These hits were further investigated for their effect on TRAIL ligand-induced caspase activation. Both siRNAs targeting IKBKE significantly reduced the activity of Caspases -8,-9 and -3/7 in TRAIL treated cells compared to siNeg transfected cells. Only one of the two siRNAs targeting each of Sharpin and LOC375449 significantly reduced the activity of the caspases, although those that did reduced the activity to a level comparable with cells not exposed to TRAIL ligand. Further, the siRNA that had the largest effect in the confirmation experiments correlated with the siRNA that had the largest effect in the caspase activity experiments.

The effect of knock-down of the confirmed hits on sensitivity to other apoptosis inducers was investigated. siRNAs targeting both Sharpin and LOC375449 affected the sensitivity of cells to both TRAIL and FAS ligand induced cell death, but did not have a statistically significant effect on non-ligand inducers of apoptosis, except at one concentration for one siRNA (Figure 4.10a,b, Figure 4.11a,b, Figure 4.12a,b and Figure 4.13a,b). This is in line with the finding from caspase activity assays that both genes act to reduce the level of Caspase-8 activation. However, it must be remembered that a lack of significance does not necessarily imply that there is no actual difference. Indeed, small differences between wells transfected with siRNAs targeting these genes did show a small effect at one concentration of H₂O₂ tested (50µM for Sharpin and 100µM for LOC375449). Further, in the UV experiments, the positive control had a small effect, similar to that of one of the Sharpin siRNAs, and interpretation of this experiment was complicated by problems with the viability of negative control transfected, untreated cells. Therefore, all that can be concluded is that there is no strong evidence for the involvement of these genes in the induction of apoptosis in response to these conditions.

Knock-down of IKBKE by one, but not both of the siRNAs targeting this gene had

a large and significant effect on apoptosis induction by both TRAIL, FAS and the non-ligand inducers tested (Figure 4.10c, Figure 4.11c, Figure 4.12c and Figure 4.13c). It is unclear why the effects were seen by only one siRNA, when the siRNAs had similar effects in the caspase assays (Figure 4.9), particularly since both siRNAs targeting Sharpin and LOC375449 had effects on ligand induced cytotoxicity, but differing effects on ligand induced caspase activity.

4.7.2.1 Sharpin

Sharpin was originally identified as a binding partner for the post-synaptic density protein Shank (Lim et al. 2001). Protein localisation studies in rat neurons showed that the protein localised in a punctuate pattern near synapses and co-localised with shank, although fractionation experiments relieved that significant portion of the protein was localised in the cytosol (Lim et al. 2001). As well as binding Shank, Sharpin is also able to homodimerise through its N terminal domain (Lim et al. 2001). Lim *et al* hypothesised that Sharpin acts as a scaffold protein. The interaction between Sharpin and Shank is mediated through the C-terminal domain, which contains a RanBP type zinc finger domain (Pfam, <http://pfam.sanger.ac.uk>) and is homologous to the N terminal domain of the Protein Kinase C binding ubiquitin ligase RBCK1 (Lim et al. 2001).

Strangely considering its inclusion in this targeted siRNA set, Sharpin contains neither kinase nor phosphatase domains. Its inclusion here in the kinase, phosphatase and kinase/phosphatase associated gene set could be due to its similarity to a kinase binding protein. Alternatively Pfam reports a “kinase-like” domain at the N terminal end, although BLAST does not reveal similarity to any kinases in this domain.

A recent report has demonstrated that a mutation in the mouse Sharpin gene leads to a dermatitis like phenotype, which is accompanied by a multi-organ inflammatory response and various immune cell and cytokine defects (Seymour et al. 2007).

The GNF expression atlas (<http://symaltas.gnf.org>) contains expression profiles for a large number of transcripts in a range of tissue types and in number of organisms. The GNF atlas profile for Sharpin reveals high mRNA expression in testis associated tissues and in the heart. This correlates with the finds of Lim *et al* who found expression in a wide range of tissues, but strong expression in the heart and testis. However, protein expression did not correlate with mRNA expression with strong protein expression in the brain, lung and spleen, and more modest expression in heart, liver, muscle, kidney and testis tissue (Lim et al. 2001).

Although no interaction has been reported between protein kinase C (PKC) and

Sharpin, the homology of Sharpin to RBCK1 in its PKC binding domain is interesting as PKC has been implicated in the regulation of TRAIL-induced apoptosis through control of FADD recruitment to the DISC and therefore Caspase-8 activation (Harper et al. 2003). If Sharpin were to interact with, and regulate, PKC then this would fit with the finding that Sharpin regulates apoptosis at or above the level of Caspase-8 activation (Figure 4.9) and could be a clue to its involvement. Although in the absence of experimental evidence this remains purely speculative but could serve as a hypothesis for further investigation.

There was a small effect of the stronger of the two siRNAs targeting Sharpin on UV- and H₂O₂-induced cell death. It has previously been reported that death receptor knock-outs show a subtle deficiency in radiation induced apoptosis (Finnberg et al. 2005), suggesting that the integrity of the extrinsic apoptosis pathway has an effect on activity of the intrinsic pathway.

4.7.2.2 LOC375449 (MAST4)

LOC375449 has recently been renamed Microtubule Associated Serine Threonine kinase 4 (MAST4) due to its similarity to a family of MAST kinases (MAST1, MAST2, MAST3 and MASTL) (Sun et al. 2006). These kinases, MAST4 included, have a conserved domain structure, with a conserved domain of unknown function at the N terminal end, followed by a kinase domain, a kinase C terminal domain and a PDZ domain (Pfam, <http://pfam.sanger.ac.uk>). PDZ domains are involved in protein/protein interactions and in MAST1-3 are involved in the binding of these kinases to the tumour suppressor PTEN (Valiente et al. 2005). However it was reported that this is not the case for MAST4. MAST1 and MAST2 are also involved in regulation of TNF α mediated activation of NF- κ B, through their phosphorylation of TRAF6 and its subsequent ubiquitination and degradation by the proteasome (Xiong et al. 2004).

The GNF atlas reports a generally even expression across tissue types, with perhaps a slight increase in expression in immune cells. Oncomine (<http://www.oncomine.org>) is a database containing the results from microarray transcription profiles for cancer cells. Oncomine reports 11 independent studies where MAST4 has a significantly increased expression in Estrogen Receptor positive breast cancer samples compared to Estrogen Receptor negative breast cancer samples. The Estrogen Receptor is a ligand activated transcription factor that has long been associated with breast cancer and several breast cancer treatments such as tamoxifen function by targeting this receptor (reviewed in Ali, Coombes 2000). A study of the effects of transfection of known oncogenes into primary human

mammary epithelial cells revealed that MAST4 was strongly up-regulated when cells were transfected with activated H-Ras (Bild et al. 2006).

The COSMIC database contains details of somatic mutations in cancer cells lines (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>). Currently the database focuses on results from systematic resequencing of kinases from a large range of cancer cell lines and primary cancer samples. The database lists six mutations in the MAST4 gene, 3 missense substitutions, 2 nonsense substitutions and one complex substitution resulting in a missense and a nonsense substitution. None of these mutations are in the kinase domain, or any other Pfam domain, as would be expected for an activating mutation. Two of these mutations (E784K and E2276*) were found in an Estrogen Receptor positive primary breast cancer sample shown to have a mutator phenotype (Stephens et al. 2005). Analysis of the spectrum of mutations found suggested that while some of the mutations found in this sample probably contributed to the oncogenic phenotype, the majority were “passenger mutations” and did not contribute to the phenotype.

Since MAST4 is a member of a protein family, it is worth considering the possibility that the siRNAs designed to target MAST4 are also targeting related genes from the same family. Pairwise alignment of the sequence of the MAST4 transcript targeted by the siRNAs used in this study to each of the other 4 members of the MAST family showed that MAST4 shares 39.9%, 49.8%, 40.91%, and 34.3% sequence similarity to MAST1, MAST2, MAST3 and MASTL respectively. When deciding if it is possible that MAST4 siRNAs target other members of the MAST family the alignment of the siRNA to the sequence is of more importance than the global alignment of the two targeted sequences. The best alignment between each of the MAST4 siRNAs is shown in Table 4-7. Thus it seems unlikely that these siRNAs are targeting these transcripts. This is not unexpected since siRNA design includes a specificity check for sequences that match this siRNA with a high sequence identity.

<i>siRNA</i>	<i>MAST1</i>	<i>MAST2</i>	<i>MAST3</i>	<i>MASTL</i>
siLOC375449.1	12/21	13/21	14/21	12/21
siLOC375449.2	14/21	15/21	14/21	12/21

Table 4-7 – Best matches of MAST4 siRNAs against other members of the MAST family

Sequences of MAST4 family members were retrieved from the ReqSeq database and aligned to the sequences of MAST4 siRNAs using a smith waterman algorithm.

Here it has been shown that MAST4 is involved in the regulation of sensitivity of cells to ligand mediated apoptosis at or above the level of Caspase-8 activation. Expression experiments seem to suggest that it is downstream of RAS (Bild et al. 2006), which is of interest as activation of the RAS pathway is known to be involved in the sensitisation of cells

to TRAIL-mediated apoptosis (see 1.3.2.6). It has also been shown to be overexpressed and mutated in estrogen receptor positive breast cancer samples.

4.7.2.3 IKBKE

The IKBKE gene codes for the Inhibitor of kappa B Kinase ϵ protein (IKK ϵ , also known as IKK-i). Upon stimulation IKK ϵ phosphorylates Inhibitor of kappa B alpha (I κ B α) targeting it for degradation and subsequently leading to activation of NF- κ B (Shimada et al. 1999). IKK ϵ has also been shown to regulate the constitutive activity levels of NF- κ B by direct phosphorylation of both the cRel and RelA NF- κ B subunits (Adli, Baldwin 2006, Harris et al. 2006). IKK ϵ is also known to directly regulate the interferon anti-viral pathways by the phosphorylation and activation of the IRF-3 and IRF-7 (Fitzgerald et al. 2003, Sharma et al. 2003). Both the NF- κ B and innate antiviral responses are known to be involved in the regulation of TRAIL sensitivity (see 1.3.2 and 1.3.4). IKBKE amplification has recently been shown to be important in breast cancer oncogenesis (Boehm et al. 2007).

In addition to a kinase domain the IKK ϵ protein, like Sharpin, contains a ubiquitin like domain (Pfam, <http://pfam.sanger.ac.uk>). This domain has been shown to be required for the regulation of antiviral response genes (Ikeda et al. 2007). Other IKK proteins also contain ubiquitin-like domains and the IKK β ubiquitin-like domain is required for its NF- κ B activating activity (Suzuki, Nakabayashi & Takahashi 2001).

The GNF expression atlas reports that IKBKE is expressed at a low level in most tissues, but expression is particularly strong in immune related cell types, particularly T cell lineages and dendritic cells. The strongest expression is found in CD8+ T cells, which is interesting given the role of TRAIL in preventing the secondary expansion of “Helpless” CD8+ T cells (Janssen et al. 2005).

Knock-down of IKBKE by siIKBKE.1 reduces sensitivity to not only TRAIL-induced apoptosis, but also to FAS-, H₂O₂- and UV--induced cell death. Caspase assays showed that IKBKE acts at or above the level of Caspase-8 activation. The effect on H₂O₂- and UV-induced cell death could be mediated by the same action of Caspase 8 on the intrinsic death pathway mentioned above in connection to the effect of Sharpin knockdown on UV- and H₂O₂-induced cell death. However the effect of IKBKE knockdown is much stronger than the effect of Sharpin knockdown. If IKBKE is involved both the NF- κ B activation, which it self has multiple effects on apoptosis regulatory proteins, and the interferon response, it is possible that IKBKE is involved in regulation of apoptotic pathways at multiple points.

4.7.3 Conclusions

In the previous chapter an assay for sensitivity to TRAIL-induced cytotoxicity was developed and used to compare different methods for performing RNAi-mediated screens for genes which affect the TRAIL-induced cytotoxicity pathway. Here the findings from that work were applied to a screen of 897 kinases, phosphatases and kinase/phosphatase associated genes. The aims of this study were two fold. Firstly the screen acts as a gene discovery exercise in its own right. Secondly the relatively small screen acts as a pilot for larger screens.

siRNAs targeting 897 kinases, phosphatases and kinase/phosphatase associated genes were transfected into HeLa cells and the sensitivity of cells to TRAIL determined. Each gene was targeted by two siRNAs and 2.75 replicates of the screen were performed, which was later condensed into 2 “high-quality” replicates. The survival of cells in each well was determined. Data was normalised using the plate median and scored by standardising the normalised survival and selecting the minimum of the two replicates as the score for each siRNA.

The correlation between the two replicates ($r = 0.65$) was much stronger than the correlation between the scores of different siRNAs targeting the same gene ($r=0.20$). This was reflected in the finding that seven of the top ten siRNAs retested for confirmation showed a significant effect on TRAIL-induced cytotoxicity, but only in three cases did additional siRNAs targeting these genes also significantly reduce the sensitivity of cell to TRAIL. The difference in the proportion of either genes or siRNAs selected on the basis of both siRNAs targeting a gene scoring higher than a threshold is not statistically different. However, selecting genes using the single high-scoring siRNA method, gave a higher number of candidates, and thus a higher number of eventual hits. The two-hit method is more restrictive, and risks missing strong hits where one siRNA has failed due to poor siRNA design, or for technical reasons. Selecting genes for follow up on the basis of two siRNAs targeting the gene scoring higher than a threshold determined using the positive controls did not successfully identify any additional genes which could be confirmed as having a role in TRAIL-induced cytotoxicity, and thus fails to increase the rate of confirmation despite the increased restrictiveness of the candidate selection. More candidates could be selected by reducing the threshold, yet this would almost certainly reduce the confirmation rate. Thus the screen is accurate at selecting siRNAs with an effect on the phenotype, but less accurate at selecting genes that are involved. Additionally, only one siRNA targeting genes previously implicated in TRAIL-induced apoptosis scored higher than this threshold (in addition to the

positive controls).

In both identifying previously associated genes and identifying novel genes (as opposed to siRNAs) the screen was clearly less successful than might have been hoped. However, while the screen clearly has not identified all genes in the set which are involved in the pathway, several high confidence genes have been isolated.

Taken together, these results suggest that the screening system described here can be successfully used to identify siRNAs which affect sensitivity to TRAIL-induced cytotoxicity. Rigorous follow up of these siRNAs can allow for the identification of novel genes involved in the pathway. They demonstrate that a follow up strategy based on following the top siRNA hits is at least as good as and possibly better than following genes that are hit by two weaker siRNAs. The screen presented is less successful at defining the absolute involvement of any one gene in TRAIL-induced apoptosis. These results emphasize the importance of careful confirmation of hits from siRNA screening experiments.

A scale up of this experiment to a larger gene set could be expected to produce a similar standard of results. Although it is unlikely that all or genes involved in the process would be isolated from a genome-scale screen, novel targets that would not have been otherwise isolated should be identified.

Three genes with a novel involvement in the TRAIL-induced apoptosis pathway have been identified. Their involvement in TRAIL-induced apoptosis as well as TRAIL-induced cytotoxicity was demonstrated using luminescent caspase activity assays, which also showed that these genes acted at the level of Caspase-8 activation. Study of the effects of knock-down of these genes on other inducers of apoptosis demonstrated that Sharpin and MAST4 are involved in ligand induced apoptosis while IKBKE is involved more generally in regulation of apoptosis, possibly acting at multiple points in the pathway. Little is known about both Sharpin and MAST4, although mutation of Sharpin has been implicated in a spontaneous inflammation phenotype in mice (Seymour et al. 2007) and MAST4 is over-expressed in estrogen receptor positive cancers and in response to RAS activation (Bild et al. 2006), and is mutated in some breast cancers samples (Stephens et al. 2005). IKBKE is involved in regulating the constitutive levels of NF- κ B (Adli, Baldwin 2006, Harris et al. 2006), a transcription factor with multiple effects on TRAIL sensitivity and also in the regulation of anti-viral responses, which is interesting as virally infected cells are sensitive to TRAIL-induced apoptosis.

5 AN siRNA SCREEN OF THE DRUGGABLE GENOME

The previous chapter presented a screen of kinases, phosphatases and associated genes for genes, that when knocked down, reduced the sensitivity of cells to TRAIL-induced apoptosis. This screen served as both a gene discovery experiment in its own right and as a pilot for larger screens. The results from this screen showed that while the methods developed were insufficiently sensitive to allow detection of the genes previously associated with sensitivity of cells to TRAIL-induced apoptosis other than the controls included on

each plate, novel genes connected to the regulation of the sensitivity of cells to TRAIL-induced apoptosis could be identified.

This chapter describes a screen of siRNAs targeting a further 6095 genes designated members of the “druggable genome”, in order to identify further genes which play a role in regulating TRAIL-induced apoptosis. Like genes identified in the kinase and phosphatase screen, the involvement of genes targeted by siRNAs scoring highly in this screen was rigorously confirmed. An exploration of possible off target effects by examination of the seed sequences of highly scoring siRNAs is also described.

5.1 The druggable genome

Screening libraries of siRNAs targeting genome subsets, such as the kinase and phosphatase library screened in the previous chapter can be useful for identifying new genes in pathways. However, such screens are based on some hypothesis about genes likely to be involved in the process, and therefore risk missing genes in the pathway. Since these genes will be genes in unexpected gene families they are more likely to point to novel aspects of biology. The ideal solution is to screen libraries targeting each gene in the genome. However, whole genome libraries remain out of the reach of all but the largest research groups, pharmaceutical companies and specialised facilities. The cost of a whole genome screen is not just limited to the, already prohibitive, cost of the library itself, but also the cost executing the screen.

The first mention of the term ‘Druggable Genome’ in Medline is in 2002, in a review by Hopkins and Groom (Hopkins, Groom 2002) who use it to describe the set of genes containing protein domains which can bind small molecules (i.e. potential drugs), although Drews referred to a hypothetical set of proteins, related to disease genes, that could be targeted by pharmaceuticals in 2000 (Drews 2000). The number of genes classified as belonging to the druggable genome varies, with published estimates being 3,000-6,000 depending on the definition and the data set used. The druggable genome generally contains GPCRs, transcription factors, kinases, phosphatases, nucleotide binding proteins, proteases and more. Thus as well as the protein products of such genes being of interest to the pharmaceutical industry as possible drug targets, the druggable genome also contains many of the information carrying and processing gene families in the genome. This makes the druggable genome an attractive choice for RNAi screening – it is small enough for purchase and use to be within the reach of a single academic group, yet contains many of the genes which could be of interest, and genes identified in this manner may be of therapeutic value.

As such, it represents a good compromise between an unbiased and a more targeted approach to screening.

The Qiagen Druggable Genome siRNA Set v2 contains siRNAs targeting 6,992 genes classified as being of “therapeutic value”. This includes the 897 kinases and phosphatases screened in the previous chapter. The composition of the library by protein family is shown in Figure 5.1.

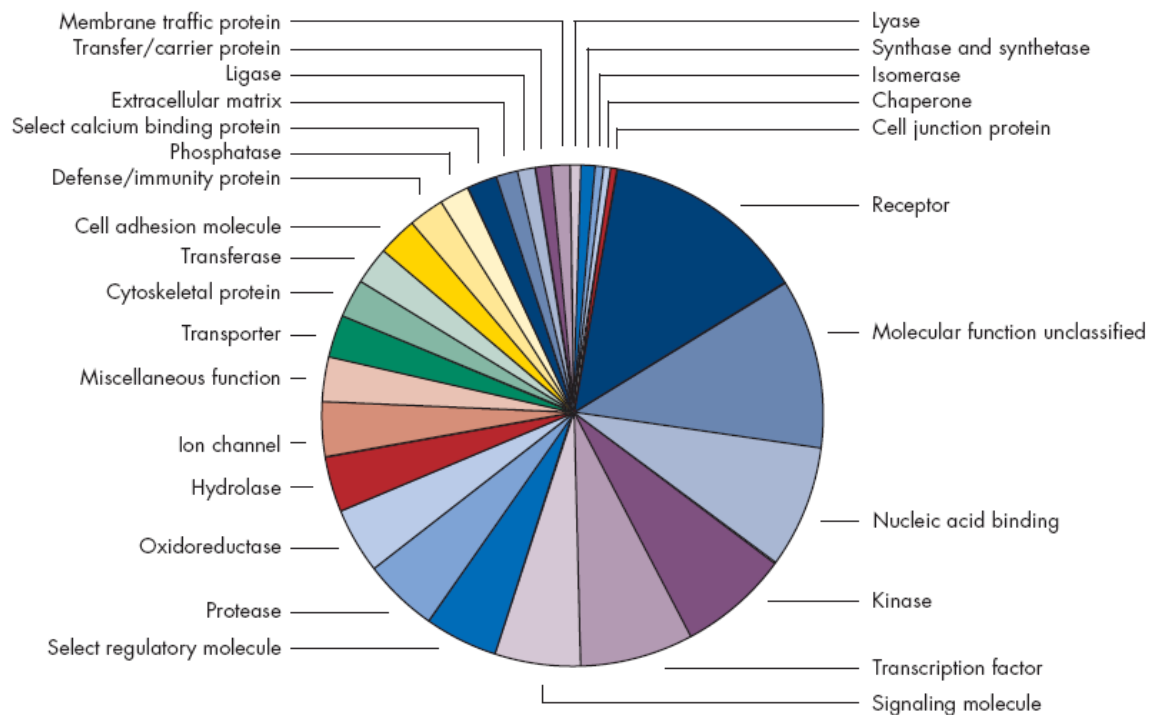


Figure 5.1 Composition of the Qiagen Human Druggable genome siRNA Set V2.0 by gene family
From Qiagen publicity material

5.2 Screen execution and initial data processing

In order to screen the library for siRNAs which affected sensitivity to TRAIL-induced cytotoxicity, siRNAs targeting 6095 genes (the genes screened in the previous chapter were not repeated) with 2 siRNAs per genes, from plates 1a – 77b of the Qiagen Druggable Genome Library v2, along with control siRNAs were transfected into HeLa cells in batches of 12 plates and the sensitivity of cells to 0.5 μ g/ml TRAIL determined. In assay development experiments, experiments were conducted using 1 μ g/ml TRAIL. Blind pseudo-screening using siRNAs gave a Z' -factor of 0.46 (Figure 3.12). In the screen reported in the previous screen comparison of the negative control with wells transfected with siCasp8 gave a Z' -score of -0.35 on a screen-wide basis. Despite the fact that this screen-wide score is calculated on the basis of plate-normalised values, it is still possible that plate-to-plate variation makes up some of this difference, since assay development pseudo-screens were

carried out on a single plate. While two wells per control are strictly insufficient to derive plate-by-plate Z' -factors, doing so gives a mean Z' -factor of 0.015 (median 0.22), showing a decrease even considering results within single plates. Except for the increase in the number of plates involved, the other difference between assay development experiments was a reduction in the concentration of TRAIL used from 1 $\mu\text{g}/\text{ml}$ to 0.25 $\mu\text{g}/\text{ml}$. In an attempt to counteract this decrease, the concentration of TRAIL used in this screen was increased to 0.5 $\mu\text{g}/\text{ml}$. In dosage curves of the effect of TRAIL on cells transfected with non-silencing siRNA, 0.5 $\mu\text{g}/\text{ml}$ TRAIL had a similar effect on survival to 1 $\mu\text{g}/\text{ml}$ (29% survival with 1 $\mu\text{g}/\text{ml}$ TRAIL compared to 31% survival with 0.5 $\mu\text{g}/\text{ml}$ TRAIL and 37% survival with 0.25 $\mu\text{g}/\text{ml}$, Figure 3.11). The layout of siRNAs on the plate used was as described in Figure 4.1. The screen was initially carried out in duplicate. After both replicates were complete plate dynamic ranges were calculated as the ratio of the geometric mean of survival in siCasp8 control transfected wells to the geometric mean of survival in negative control transfected wells. Plates with a dynamic range of less than 2 were repeated. The repeated plate dynamic range was compared to the dynamic ranges of the two original replicates. The two of the three replicates with the largest dynamic range were used. In total each replicate of the screen took approximately 3 weeks to complete. The resulting 58,136 data points were analysed using the R/Bioconductor package cellHTS

It was previously observed that sensitivity of HeLa cells to TRAIL is dependent on the density of cells. Variation in density of cells could be due to two factors. Firstly variation could be due to inaccuracies in dispensing cells into the assay plates. Secondly variation in density of cells at time of treatment could be due to effects of particular siRNAs on the viability of cells. In order to investigate the relationship between pre-treatment viability and sensitivity of cells to TRAIL-induced cytotoxicity, pre-treatment viability was normalized to the plate median pre-treatment viability to account for plate to plate variations and the normalized viabilities divided into 20 quantiles. Post-treatment survivals were normalised to the plate-median survivals, and the median normalized survival for each viability quantile was calculated. Figure 5.2 shows the relationship between pre-treatment viability and post treatment normalized survival (blue line). There is no strong relationship between viability and survival when considering the higher viability quantiles. However, at lower viabilities there is a strong relationship between pre-treatment viability and sensitivity to TRAIL-induced cytotoxicity: cells in wells with lower pre-treatment viabilities are more sensitive to TRAIL-induced cytotoxicity. Since data is normalised on a per-plate basis, with the assumption that the median survival on the plate represents an estimation of base-line

sensitivity to TRAIL, wells where TRAIL sensitivity is high due to a low pre-treatment viability can affect plate normalisation factors. In order to prevent the observed trend affecting further analysis for this reason, wells in the bottom 20% for pre-treatment viability were removed from further analysis. Applying this cut-off entirely negated the relationship between pre-treatment viability and sensitivity to TRAIL-induced cytotoxicity (Figure 5.2, red line).

Data from each plate was normalized to the median of the survival in wells

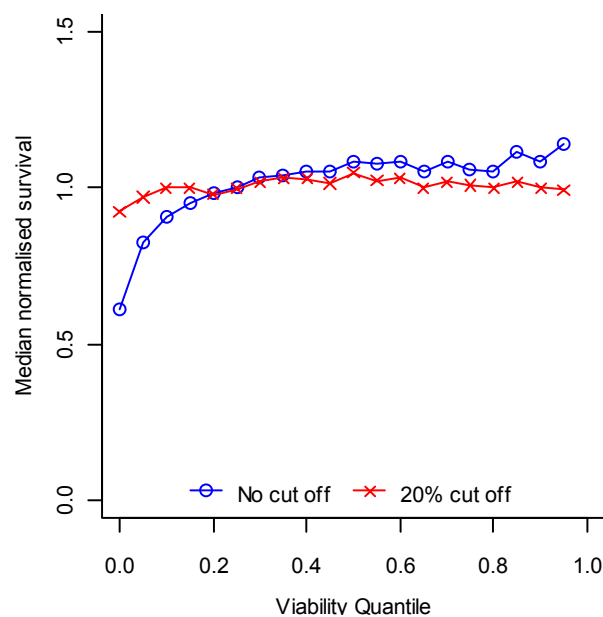


Figure 5.2 Relationship between pre-treatment viability and sensitivity to TRAIL-induced cytotoxicity
Viability of cells in each well prior to treatment was normalized to plate median viabilities. Normalised viabilities were divided into 20 quantiles. The median normalized post treatment survival was calculated for wells in each of these quantiles. The blue line represents the relationship between pre-treatment viability and post-treatment normalized survival for the entire data set. The red line represents the same relationship with the 20% of wells with the lowest pre-treatment viabilities are removed from the analysis.

containing “sample” siRNAs for each plate. Figure 5.3 shows effects of this normalisation. The raw data from the screen is very variable (Figure 5.3a), with the minimal survival value on some plates being higher than the maximum on other plates. Plate-to-plate variation is probably higher in this screen compared to the kinase and phosphatase screen due to the higher number of different batches of cells required to complete the screen, with each replicate of the kinase and phosphatase screen being completed with a single batch of cells, while each replicate of the screen presented here required several independent batches of cells, with for example, replicate 2 of the screen using cells from 10 flasks, defrosted from liquid nitrogen on 3 separate dates.

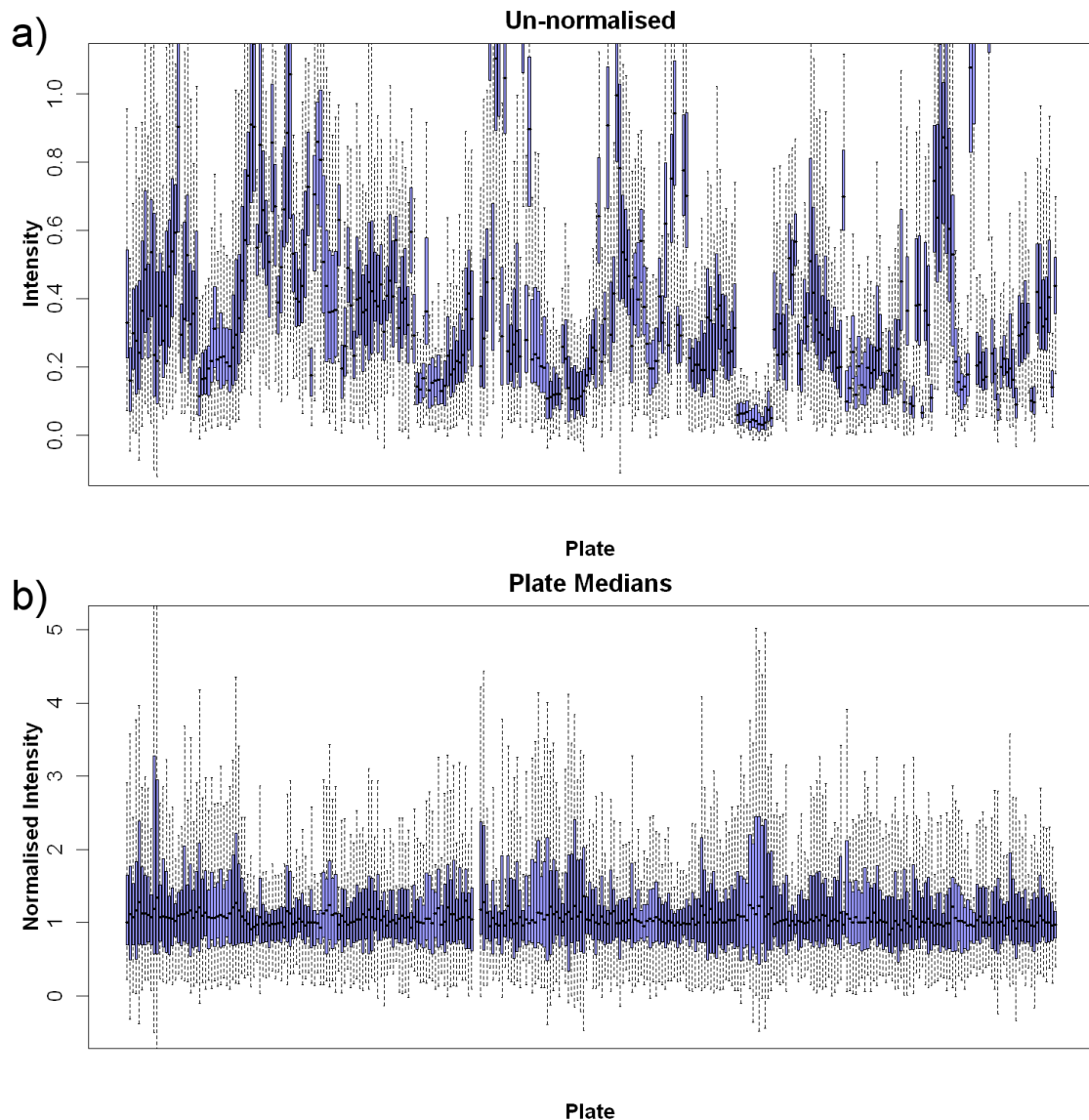


Figure 5.3 Normalisation of data from screen of druggable genome.

a) Box-plot of raw survival data from screen on a per plate basis. b) Box-plot of survival data normalized to plate median survival on a per plate basis

A correlation between effect size and variability can complicate interpretation of results. Such a correlation was observed in the data from the kinase and phosphatase screen. Various transformations, such as log transformation, can help to remove such correlations. The rank of the mean normalized survival for the replicates of each well was plotted against the standard deviation in order to examine a possible relationship between the two (Figure 5.4). A clear relationship between the mean and standard deviation can be observed in non-transformed data (Figure 5.4a): an increase in mean is accompanied with a large increase in standard deviation at the higher ranks. A relationship can also be observed between rank of mean and standard deviation for log-transformed data (Figure 5.4b): standard deviation is higher at lower ranks. However, the relationship does not appear to be as strong in log

transformed data. Observing the running median line, shown in red, indicates a stronger relationship, over a larger portion of the results for non-transformed data. On this basis, further analyses were conducted using log transformed values.

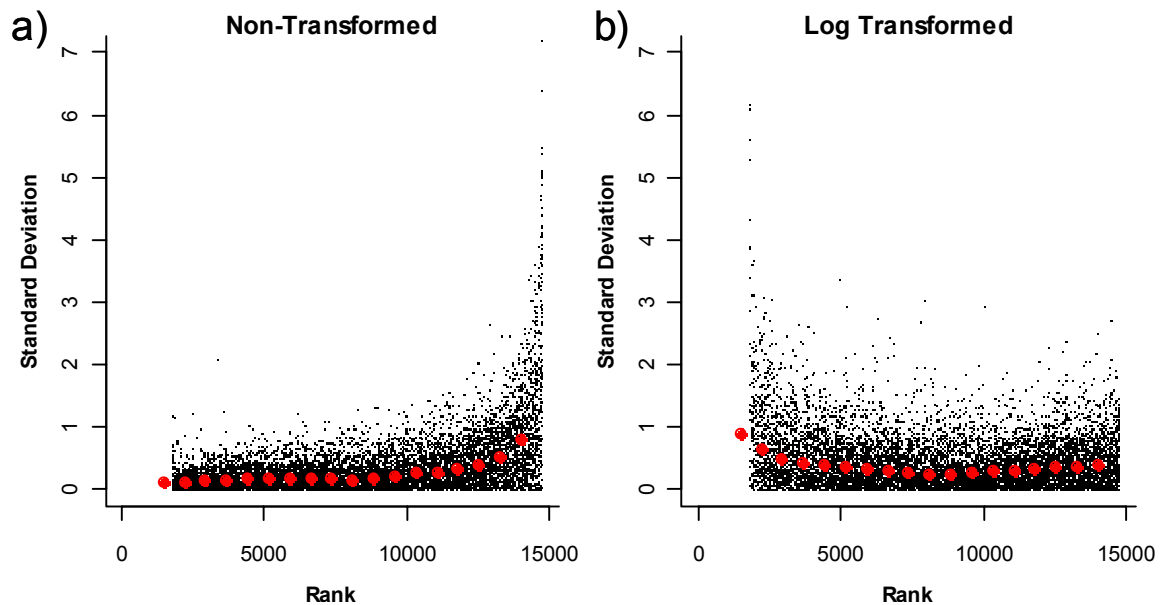


Figure 5.4 Relationship between standard deviation and rank of the mean for siRNAs.

For each siRNA the mean of the normalized data of the two replicates was calculated. The rank of this mean was then plotted against the standard deviation between the replicates for a) Non-transformed data and b) Log-transformed data. The red line in each plot represents the running median standard deviation.

Data were analysed using cellHTS, first excluding wells with a low pre-treatment viability, and then median normalizing plates with a log transformation, and using the minimum of replicates as a summary function. The HTML reports produced can be found on the included CD, or online at http://www.sanger.ac.uk/HGP/Chr22/RNAi/TRAIL_DG

5.3 Screen quality and analysis of controls

The processed screening data was used to assess the quality of the screen (Figure 5.5). The Pearson's correlation co-efficient, r , between the two replicates was 0.57 (Figure 5.5a), similar to that found in the kinase and phosphatase screen (0.65). As in the kinase and phosphatase screen the correlation between the two siRNAs targeting the same gene was dramatically lower at $r = 0.075$ (Figure 5.5b). Once again this demonstrates that the effect of a particular siRNA on the sensitivity of cells to TRAIL-induced cytotoxicity is fairly reproducible, while, the targeting of the same gene with different siRNAs does not give a reproducible effect.

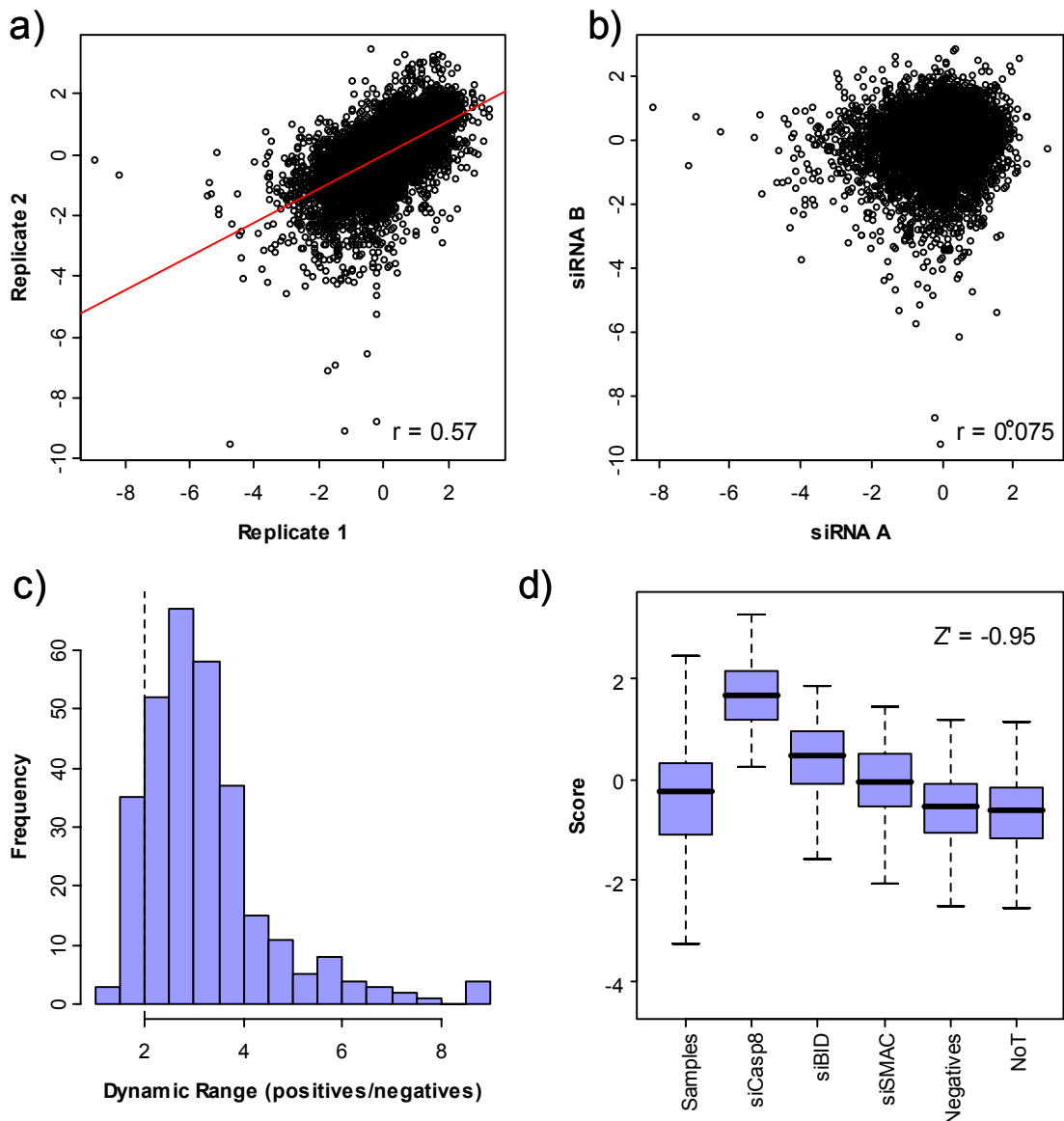


Figure 5.5 Assessment of screen quality and controls.

a) Normalized survival from replicate 1 plotted against normalised survival from replicate 2. Red line shows linear regression of replicate 2 on replicate 1. The Pearson's correlation co-efficient is shown in the bottom right corner. b) Plot showing normalised survival of the two siRNAs targeting the same gene. The Pearson's correlation co-efficient is shown in the bottom right corner. c) Histogram showing the distribution of plate dynamic ranges (see above for definition of plate dynamic range). Dashed line represents a dynamic range of 2. d) Box plot summarising the scores in different well types. Z²-factor between negative controls and siCasp8 is shown in top right corner. NoT = Untransfected

Figure 5.5c shows the distribution of plate dynamic ranges (the ratio of the geometric mean of survival of siCasp8 transfected wells and geometric mean of survival of negative control transfected wells). Despite increasing the concentration of TRAIL for this screen, the majority of plate dynamic ranges are still between 2 and 4 (70%, Figure 5.5c), with a minority having a dynamic range less than 2 (12%) and greater than 4 (18%).

The distribution of scores for different well types is shown in Figure 5.5d and Table 5-1. The median survivals, and consequently the median scores are higher for all of the positive controls than the negative controls. The difference in raw survival between siCasp8

<i>Category</i>	<i>Median Score</i>	<i>Median Survival</i>	<i>Survival MAD</i>
Samples	-0.23	28.2%	23.5%
siCasp8	1.68	80.5%	40.4%
siBID	0.48	48.4%	28.6%
siSMAC	-0.03	37.1%	21.0%
Negatives	-0.54	27.3%	18.1%
Untransfected	-0.59	27.9%	19.2%

Table 5-1 Summary statistics for different well types in screen of druggable genome

and the negative controls is larger than in the kinase and phosphatase screen. However the difference in score is much lower, due, both to the greater variance in this screen, and the log transformation of the data. Wells transfected with siBID and siSMAC are intermediate between siCasp8 and the negative controls. The Z' -factors for the difference between siCasp8, siBID, siSMAC and the negative controls are -0.95, -3.44 and -7.52 respectively. Again the increase in throughput has led to a reduction in the Z' factors, due to the increase in the variability. This indicates that there would be little chance of finding hit with effects smaller than those of BID or SMAC, and a reduced chance of finding even hits with an effect size similar to Caspase-8.

The scores and median survivals of untransfected wells (-0.59 and 27.9% respectively) are remarkably similar to those for the negative controls (-0.54 and 27.3% respectively) and the spread of values is similar. The median score for sample wells is slightly higher than those for the negative controls (in contrast to the results of the kinase and phosphatase screen, where the negative controls had a higher median score), but the difference in terms of median survival is very small.

5.4 Screen Results

In the screen of kinases and phosphatase the scores from sample wells formed a distribution with an elongated left-hand tail and a foreshortened right-hand tail. Here the distribution of scores is closer to normal, but is skewed in the opposite direction due to the log transformation of the values (Figure 5.6). One interpretation of this would be that there are many siRNAs which are causing an increase in the sensitivity of cells to TRAIL-induced apoptosis. Under this hypothesis one would expect that if the results were analysed to find such siRNAs, that is, wells with a low proportion of cells surviving after TRAIL treatment were given a high score, that the distribution would be reversed, with a long tail of highly positive siRNAs. However, this is not the case with the resulting distribution of scores having a similar shape to the distribution seen here (data not shown). This suggests that the skew in the distribution of scores is an artefact of the analysis process.

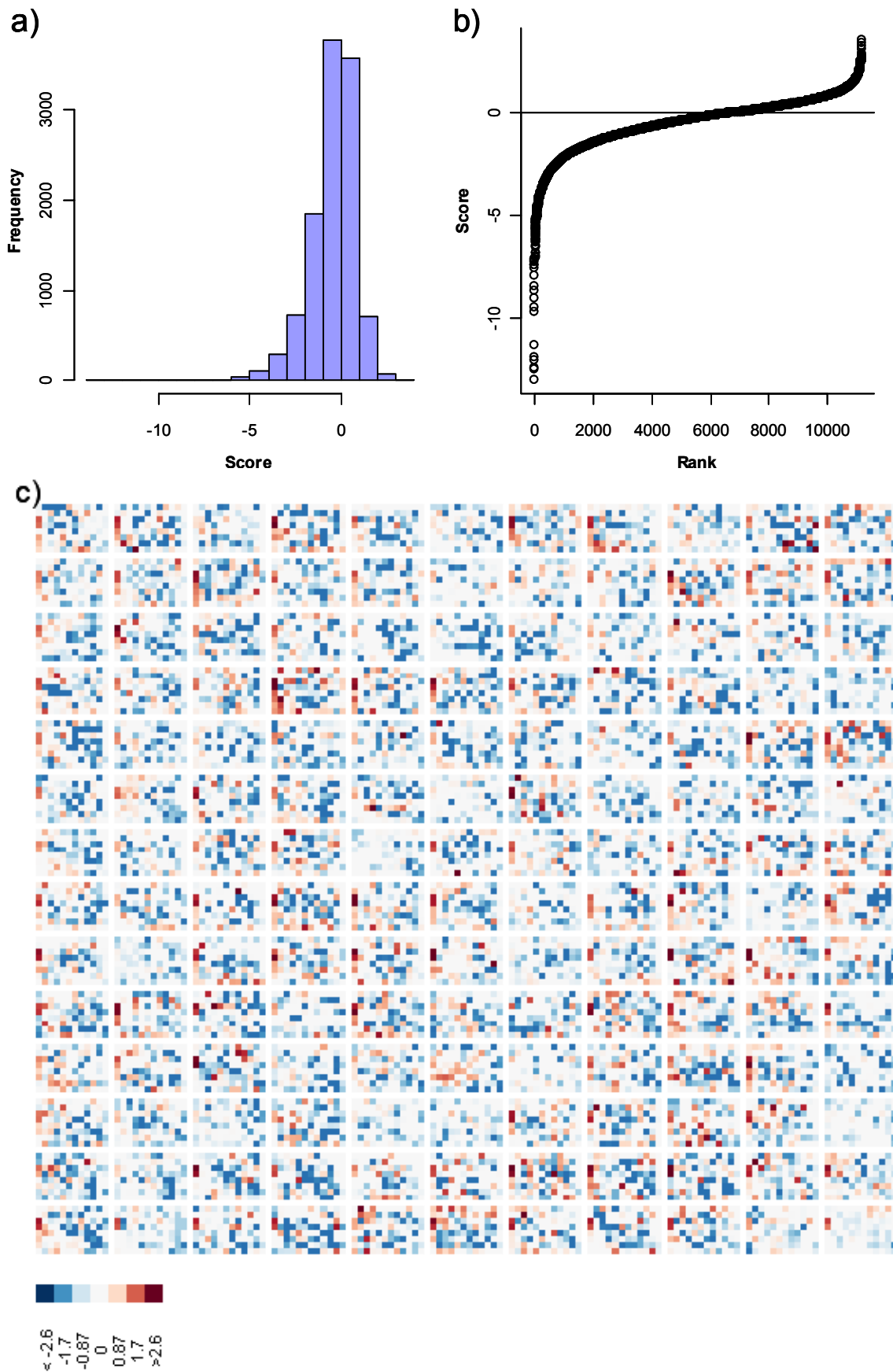


Figure 5.6 Results of siRNA screen of the druggable genome

a) Histogram of scores from sample wells b) Rank of siRNA score from sample wells plotted against score. c) Heat map of scores per plate. siRNAs with a highly positive score are shown in red, siRNAs with a highly negative scores are shown in blue. Plates are arranged row-wise.

<i>GeneID</i>	<i>Symbol</i>	<i>Description</i>	<i>Normalized Survival</i>		<i>score</i>
			<i>Rep 1</i>	<i>Rep 2</i>	
NM_003217	TEGT	Testis enhanced gene transcript (BAX inhibitor 1)	NA	2.572	3.51
NM_016368	ISYNA1	myo-inositol 1-phosphate synthase A1	2.468	NA	3.43
NM_001240	CCNT1	cyclin T1	2.361	NA	3.28
NM_002197	ACO1	aconitase 1, soluble	2.844	2.279	3.11
NM_003947	HAPIP	huntingtin-associated protein interacting protein (duo)	2.074	NA	2.88
NM_002337	LRPAP1	Low density lipoprotein receptor-related protein associated protein 1	2.018	NA	2.8
NM_005799	INADL	InaD-like (Drosophila)	2.03	2.026	2.76
NM_003554	OR1E2	olfactory receptor, family 1, subfamily E, member 2	1.962	NA	2.72
NM_013345	GPR132	G protein-coupled receptor 132	2.755	1.961	2.68
NM_004584	RAD9A	RAD9 homolog A (S. pombe)	NA	1.943	2.65
NM_003266	TLR4	Toll-like receptor 4	1.975	1.934	2.64
NM_000674	ADORA1	adenosine A1 receptor	1.893	NA	2.63
NM_002382	MAX	MAX protein	2.416	1.883	2.57
NM_006986	MAGED1	melanoma antigen, family D, 1	1.843	2.424	2.56
NM_000875	IGF1R	insulin-like growth factor 1 receptor	1.838	NA	2.55
NM_016953	PDE11A	phosphodiesterase 11A	1.865	1.865	2.54
NM_014379	KCNV1	potassium channel, subfamily V, member 1	1.832	1.911	2.54
NM_080674	C20orf86	chromosome 20 open reading frame 86	2.151	1.847	2.52
NM_002467	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	NA	1.842	2.51
XM_497793	LOC402037	similar to alpha tubulin	1.815	1.838	2.51
XM_089281	LOC149281	similar to RIKEN cDNA 2610205E22	NA	1.817	2.48
NM_001962	EFNA5	ephrin-A5	2.116	1.812	2.47
NM_138295	PKD1L1	polycystic kidney disease 1 like 1	1.754	1.881	2.43
NM_017522	LRP8	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	1.724	NA	2.39

Table 5-2 Top scoring siRNAs from screen of the druggable genome

Table shows the top 24 siRNAs ranked by score from the screen. NA indicates that result was removed due to low pre-treatment viability. The complete table is available in the file topTable.txt on the included CD or online at http://www.sanger.ac.uk/HGP/Chr22/RNAi/TRAIL_DG

Gene ID	Gene Symbol	Description	Score		Minimum Score	Maximum Score	Mean Score
			siRNA 1	siRNA 2			
NM_018558	GABRQ	gamma-aminobutyric acid (GABA) receptor, theta	1.59	1.45	1.45	1.59	1.52
NM_016368	ISYNA1	myo-inositol 1-phosphate synthase A1	1.81	1.45	1.45	1.81	1.63
NM_002357	MAD	MAX dimerization protein 1	1.61	1.43	1.43	1.61	1.52
NM_000875	IGF1R	Insulin-like growth factor 1 receptor	2.55	1.43	1.43	2.55	1.99
NM_054032	MRGX4	G protein-coupled receptor MRGX4	1.41	1.58	1.41	1.58	1.50
NM_003217	TEGT	Testis enhanced gene transcript (BAX inhibitor 1)	1.35	3.51	1.35	3.51	2.43
NM_001196	BID	BH3 interacting domain death agonist	1.33	2.01	1.33	2.01	1.67
NM_013231	FLRT2	fibronectin leucine rich transmembrane protein 2	1.78	1.33	1.33	1.78	1.55
NM_018337	ZNF444	zinc finger protein 444	1.31	2.07	1.31	2.07	1.69
NM_017949	CUEDC1	CUE domain containing 1	1.26	1.94	1.26	1.94	1.60
NM_002063	GLRA2	glycine receptor, alpha 2	1.23	1.25	1.23	1.25	1.24
NM_001279	CIDEA	cell death-inducing DFFA-like effector a	1.42	1.22	1.22	1.42	1.32
NM_198150	DKFZp313G1735	hypothetical protein DKFZp313G1735	1.20	2.11	1.20	2.11	1.66
NM_018527	NARG1L	NMDA receptor regulated 1-like	1.27	1.20	1.20	1.27	1.24
NM_198857	FLJ43855	similar to sodium- and chloride-dependent creatine transporter	1.48	1.18	1.18	1.48	1.33
NM_006340	BAIAP2	BAI1-associated protein 2	1.18	1.31	1.18	1.31	1.25
XM_377955	ANKIB1	ankyrin repeat and IBR domain containing 1	1.17	1.60	1.17	1.60	1.39
NM_018319	TDP1	tyrosyl-DNA phosphodiesterase 1	1.48	1.17	1.17	1.48	1.33
NM_020919	ALS2	amyotrophic lateral sclerosis 2 (juvenile)	1.16	1.17	1.16	1.17	1.17
NM_004821	HAND1	Heart and neural crest derivatives expressed 1	1.16	1.38	1.16	1.38	1.27
NM_018110	DOK4	Docking protein 4	1.15	1.49	1.15	1.49	1.32
NM_005856	RAMP3	Receptor (calcitonin) activity modifying protein 3	1.33	1.12	1.12	1.33	1.23
NM_005252	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	1.11	2.14	1.11	2.14	1.63
NM_019839	LTB4R2	leukotriene B4 receptor 2	1.37	1.09	1.09	1.37	1.23

Table 5-3 Extract from table summarising results of screen of the druggable genome on a per gene basis

Genes are ranked on basis of the minimum of the scores from the two siRNAs. Where no score for an siRNA can be determined as the normalized survival for each of the replicates is NA, due to low pre-treatment survival, the score for the siRNA is taken to be NA, and NA is ranked as low. Full table is available as perGene.tab on included CD or online at http://www.sanger.ac.uk/HGP/Chr22/RNAi/TRAIL_DG/perGene.tab

Examination of the rank/score plot (Figure 5.6b) shows that the distribution of scores from this screen is essentially continuous, with a large section where the rate of increase in score with rank is constant. However, this section covers a smaller proportion of the total range of scores than did the equivalent section of scores from the kinase and phosphatase screen (Figure 4.6).

Figure 5.6c shows the spatial distribution of scores within the library. There are no obvious plate position effects. High and low scores are evenly distributed between and within plates and there are no obvious signs of edge effects.

siRNAs were ranked by their score in the screen, and genes ranked by the minimum of the score of the two siRNAs targeting the gene. Portions of these rankings are shown in Table 5-2 and Table 5-3.

In analysing the results of the kinase and phosphatase screen a cut off was established for hit selection based on capturing 95% of the siCasp8 positive controls. An equivalent cut off score for this screen would be 0.51 and this would also capture 6.7% of the negative controls. However, given that using this cut off to select “hit” genes was not successful in the case of the kinase and phosphatase screen, such a cut off was not be used to select “hit” genes here.

5.4.1 Analysis of genes previously associated with the TRAIL pathway

Examination of the results from siRNAs targeting genes known to be involved in the TRAIL apoptosis pathway can be used to give an assessment of the sensitivity of the screen. Raw survivals, scores and rank in the list of siRNAs for genes previously associated with the TRAIL pathway are given in Table 5-4. Without defining a cut-off it is not possible to say how many of these genes were “hits”. Four genes are targeted by siRNAs with a score greater than 0.51 (BID, Casp8, MYC and DR4), and one is targeted by two (BID), while only one of the siRNAs targeting DR4 has a score. BID, Casp8 and DR4 are the genes that gave the largest effect when knocked down in assay development experiments. However, as previously noted, using such a cut-off, based on the results from controls did not prove a successful way of identifying hits previously.

<i>Gene Symbol</i>	<i>Survival</i>		<i>Score</i>		<i>Rank</i>	
	<i>siRNA 1</i>	<i>siRNA 2</i>	<i>siRNA 1</i>	<i>siRNA 2</i>	<i>siRNA 1</i>	<i>siRNA 2</i>
BAX	24%	72%	-0.32	-0.49	5910	6537
BID	109%	126%	1.33	2.01	396	76
CASP3	32%	73%	-0.86	-0.15	7756	5209
CASP8	137%	98%	0.87	0.43	1119	2376
DVL2	61%	79%	0.04	0.32	4177	2813
FADD	16%	NA	-0.74	NA	7365	NA
FBXO11	92%	NA	0	NA	4437	NA
MYC	66%	107%	2.51	0.44	2347	19
TCF4	17%	68%	-1.72	0.33	9590	2785
TNFRSF10A (DR4)	NA	40%	NA	0.64	NA	1641
TNFRSF10B (DR5)	36%	16%	0.07	-1.15	3967	8504
VPS16	19%	50%	-0.95	-0.86	7872	7739
Median	36%	72%	0	0.325	4437	2799

Table 5-4 Survivals, Scores and Ranks of siRNAs targeting genes previously associated with the TRAIL pathway in the screen of the druggable genome

Gene Set Enrichment Analysis (GSEA) is a method for testing if genes in a pre-defined gene set are enriched in high scores in a ranked list of genes (Subramanian et al. 2005). Enrichment scores are calculated by scanning a ranked list of genes and increasing the ‘running’ enrichment scores each time a member of the gene set is encountered and decreasing the score each time a gene which is not a member of the gene set is encountered. The amount by which the score is increased on encountering a member of the gene set depends on the value of the metric used to rank the genes. The score for the gene set is the maximum enrichment score reached during the walk across the ranked list. This score is normalised for the size of the gene set, and the significance of the normalized enrichment score calculated by using permutations of the data. In this way the statistic is roughly equivalent of a weighted Kolmogorov–Smirnov like statistic. This technique is usually applied to microarray gene expression data, comprising several arrays measuring expression levels in phenotypically positive or negative samples. In this case the phenotype labels are permuted (so called column wise permutation) and the enrichment score for each gene set re-calculated to assess the significance of the normalized enrichment scores. However, the screening data used here is not suitable for this process, comprising only four samples (two treated, two untreated). In such a case, significance can be assessed by permuting the gene list (so called row wise permutation) and recalculating the enrichment scores for each gene set.

The p-values calculated this way are then usually corrected using either a false discovery rate (FDR) estimation, or a family-wise error rate (FWER) estimation. Row-wise permutation has the disadvantage that it may under-estimate the variance of the enrichment score by breaking up any correlation between genes in the gene set, and so generally the

more conservative FWER estimation of significance rather than the FDR estimation is used for p-values calculated using row-wise permutations (A. Liberzon, GSEA team, personal communication). This algorithm is implemented in the software package GSEA-P.

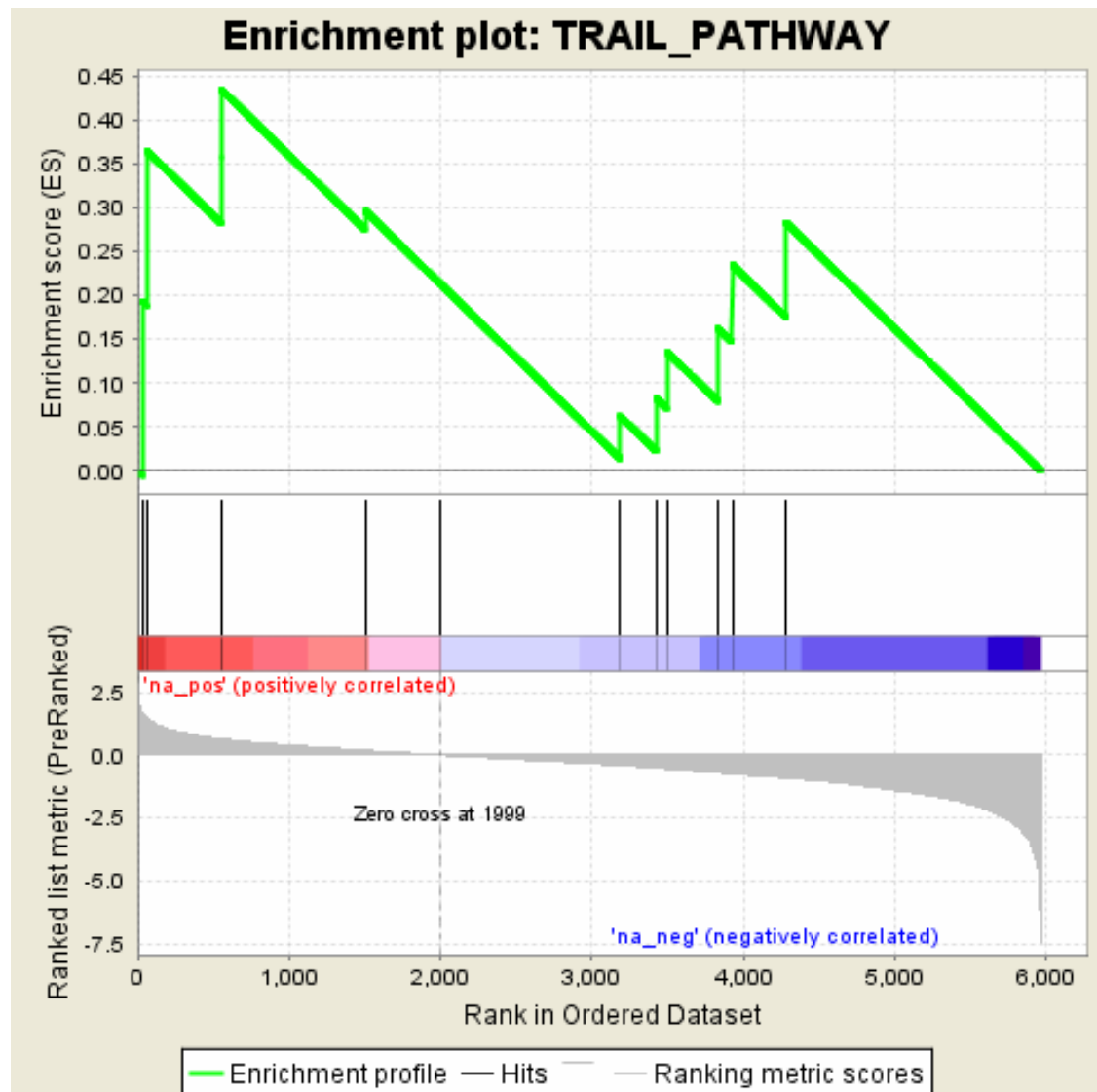


Figure 5.7 Enrichment plot for GSEA of genes previously associated with TRAIL pathway in the screen of the druggable genome

Top panel shows running enrichment score for genes previously associated with the TRAIL pathway across the ranked gene list from the druggable genome screen. The centre panel shows the position of the genes in the set in relation to the gene list and the lower panel shows the value of the ranking metric (mean score of siRNAs targeting gene).

The genes from the screen were ranked according to the average of the two siRNAs targeting the gene and GSEA-P was used to perform GSEA on the ranked list of screening results using the gene-set of genes previously associated with the TRAIL pathway as the set of genes for which enrichment is to be measured. Previously genes have been ranked on the basis of the minimum of the two siRNAs. In this case a conservative approach is taken to increase the confidence that identified genes are indeed involved in the process. Here, it is assumed that the genes are involved in the process, and so a more neutral summary of the

effect of knocking down the gene is used. The results of this analysis show that the set of previously associated genes is enriched at the top of the ranked list of genes (Figure 5.7). However, this result is only borderline significant with a nominal p-value of 0.083. The nominal p value is used rather than a corrected value since only one gene set was tested. Similar analysis of the kinase and phosphatase screen shows no enrichment for TRAIL pathway gene in the ranking of genes in that screen (data not shown).

The leading edge subset is the subset of genes that are higher in the list than the point at which the maximum enrichment score is reached and thus can be said to have contributed to the high score. In this case the leading edge subset consists of four genes: Casp8, BID, DR4 (TNFRSF10A) and MYC. This set of four genes includes three of the six “core-death pathway” genes in the set and it has been previously reported that knock-down of one of these six, DR5 (TNFRSF10B), is ineffective in preventing TRAIL-induced apoptosis in HeLa cells (Aza-Blanc et al. 2003). Of the six non-“core-death pathway” genes in the set 4 are non-confirmed hits from the Aza-blanc *et al* screen.

Thus, while it is not possible use the results here to define a sensitivity for the screen, the screen here can be shown to have been more sensitive for identifying genes previously associated with the TRAIL pathway than the kinase and phosphatase screen. This is probably at least in part due to the fact that the genes included in the screen described here from the TRAIL pathway are likely to induce a larger change in TRAIL sensitivity (e.g. Casp8, BID) compared with the TRAIL pathway genes present in the kinase and phosphatase screen and a smaller proportion of them are unconfirmed hits from the Aza-blanc screen. That is, this screen appears to be more sensitive because the sensitivity is being measured against a strong, better validated set of genes.

5.5 Confirmation of hits

In order to state that genes targeted by high scoring siRNAs are involved in TRAIL-induced apoptosis, it must be demonstrated that the effects are 1) reproducible, 2) specific and 3) related to TRAIL-induced apoptosis, rather than solely TRAIL-induced cytotoxicity. This requires that genes are targeted by multiple siRNAs that reproducibly affect TRAIL sensitivity (phenotypically active siRNAs), that these siRNAs knock-down the mRNA level of the targeted transcript to a greater extent than siRNAs which do not affect TRAIL sensitivity (phenotypically inactive siRNAs) and the activity of these siRNAs must also be reproduced in an assay that measures apoptosis rather than cytotoxicity.

Selecting genes based on a single high-scoring siRNA ultimately lead to the

identification of a larger number of confirmed high genes in the screen of kinases and phosphatases compared to selecting genes based on the score of both siRNAs targeting the gene. Therefore, here, the genes targeted by the 20 highest scoring siRNAs were selected for confirmation.

During confirmation of hits from the kinase and phosphatase screen, resources were wasted re-synthesising siRNAs which scored highly in the initial screen, but failed to repeat in confirmation experiments. Therefore, here both siRNAs targeting the selected genes from the library were retested as an initial filter. siRNAs were transfected into cells in triplicate and tested for sensitivity to 0.5µg/ml TRAIL using the alamarBlue assay Table 5-5. Transfection of 23 of the 42 tested siRNAs induced a reduction in sensitivity to TRAIL compared to transfection of the negative control which was significant at the 10% level (using a student's t-test on log transformed data, with p values corrected using the Hommel correction for multiple testing). This includes 71% of the siRNAs which were initially among the top 20 siRNAs (plus the top scoring MAD siRNA) and 38% of the second siRNAs targeting the same genes. In two cases the siRNA which was amongst the top 20 scoring siRNAs did not induce a significant change in TRAIL sensitivity in this test, while the second siRNA targeting the same gene did. Six genes were targeted by two siRNAs that significantly reduced the sensitivity to TRAIL.

At this point the four genes not targeted by any siRNA which significantly reduced the sensitivity to TRAIL-induced cytotoxicity were discarded. Where a gene was targeted by two siRNAs that significantly reduced the sensitivity to TRAIL induced cytotoxicity these two siRNAs were re-synthesised for use in future experiments. Where a gene was targeted by only one siRNA that significantly reduced the sensitivity of cells to TRAIL-induced cytotoxicity, this siRNA was re-synthesized, and in addition two further siRNAs targeting the gene were obtained.

<i>Gene</i>	<i>p-value</i> <i>siRNA A</i>	<i>p-value</i> <i>siRNA B</i>
TEGT	0.01275	0.005579
ISYNA1	0.498359	0.0164
CCNT1	0.286036	0.002418
ACO1	0.006337	0.008985
HAPIP	0.286036	0.076998
LRPAP1	0.06912	0.230341
INADL	0.498359	0.081816
OR1E2	0.498359	0.097019
GPR132	0.170937	0.006844
RAD9A	0.302649	0.00775
TLR4	0.241735	0.002145
ADORA1	0.261679	0.054469
MAX	0.002621	0.01308
MAGED1	0.923002	1
IGF1R	0.054469	0.008152
PDE11A	0.362945	0.056278
KCNV1	0.238429	0.498359
C20orf86	0.461725	0.462106
MYC	0.010032	0.002418
LOC402037	0.286036	0.286036
MAD	0.006964	0.008152

Table 5-5 Re-screen of siRNAs targeting genes targeted by the top 20 siRNAs from the druggable genome screen

siRNAs from the library targeting genes which were targeted by the top 20 scoring siRNAs from the druggable genome screen were transfected, along with siNeg, into HeLa cells in triplicate and these cells were tested for their sensitivity to TRAIL-induced cytotoxicity. p-values were calculated by one-tailed student's t-test on log transformed data and corrected using a Hommel correction for multiple testing. P-values less than 0.1 are highlighted in bold. Genes are ranked by the score of the highest ranking siRNA in the screen

In order to confirm the involvement of the selected genes in TRAIL apoptosis, and also to test the activity of novel siRNAs obtained, the effect of transfection of the siRNAs on TRAIL-induced Caspase-3/7 activity was measured using a luminescent Caspase-3/7 assay (Figure 5.8a). Transfection of siCasp8 reduced the level of TRAIL-induced Caspase-3/7 activity to 44% and 41% (for plates 1 and 2 respectively) compared to levels in cells transfected with the negative control. Transfection of 29 of 46 siRNAs significantly reduced the level TRAIL-induced Caspase-3 activity compared to the negative control. This includes 20 of the 22 siRNAs previously shown to have a significant effect on TRAIL-induced cytotoxicity. In total ten genes were targeted by at least two independent siRNAs that caused a significant reduction in TRAIL-induced Caspase-3/7 activity and two genes were targeted by three independent siRNAs that caused a significant reduction in TRAIL-induced Caspase-3/7 activity.

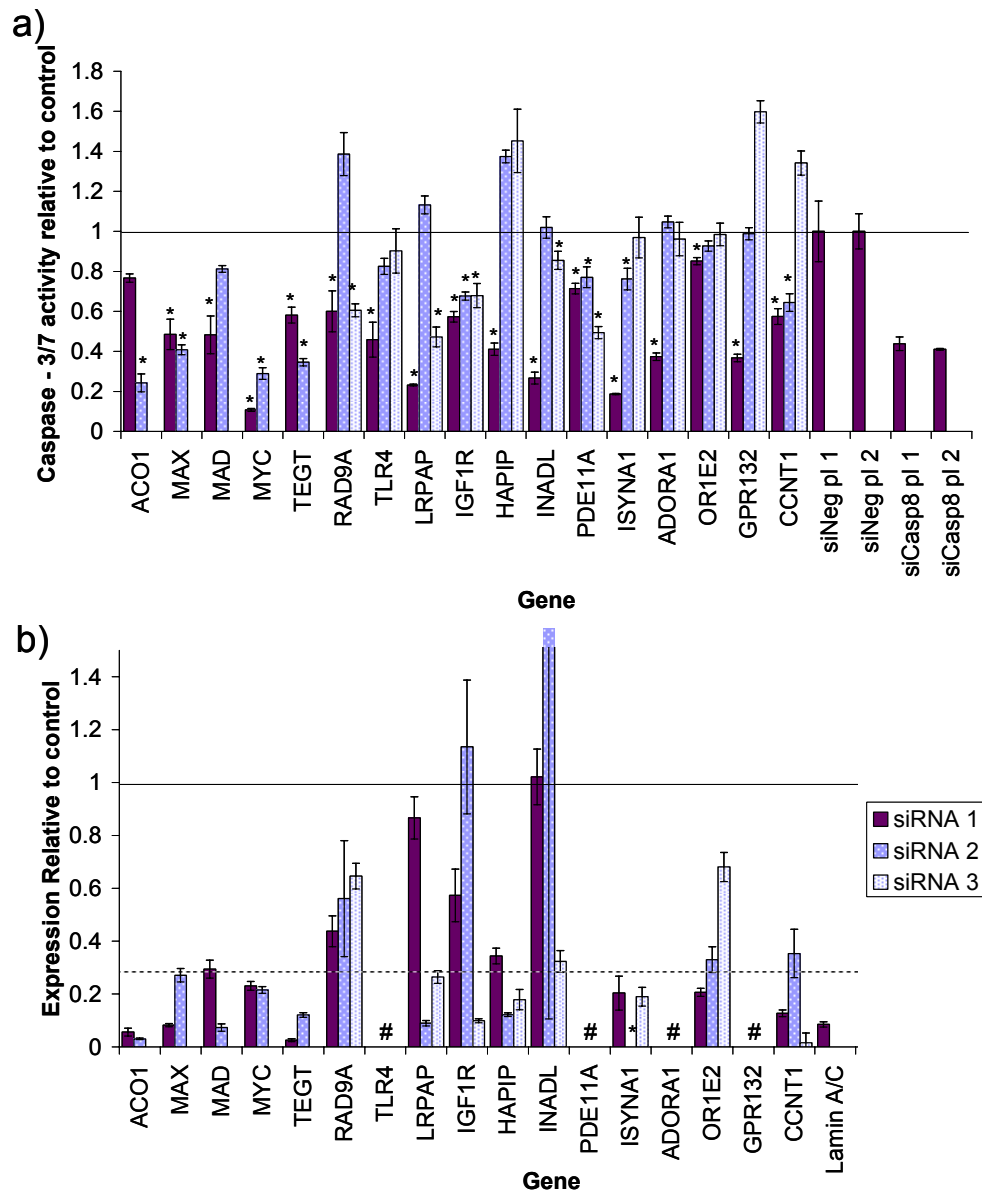


Figure 5.8 Confirmation of candidate hits from a screen of the druggable genome

a) Effects of transfection of siRNAs targeting candidate hit genes on TRAIL-induced Caspase-3/7 activity. siRNAs targeting candidate hit genes, Caspase-8, or a negative control were transfected into HeLa cells in triplicate. Cells were treated with 0.5 μ g/ml TRAIL for 6 hours, 48 hours after transfection. Caspase-3/7 activity was measured using Promega's Caspase-Glo 3/7 assay. The experiment was conducted over two plates. Each plate included siNeg and siCasp8 control transfections. siNeg pl1 and siCasp8 pl1 are controls from plate 1, and siNeg pl2 and siCasp8 pl2 are controls from plate 2. Results are normalised to control levels. Solid line represents 100% of control activity. * result is significantly different, at the 5% level, from the negative control of the same plate. P-values calculated using student's t-test. b) Effect of transfection of siRNAs targeting candidate hit genes on mRNA levels of targeted gene. cDNA was prepared by reverse transcription of RNA isolated from cells transfected with siRNAs targeting candidate hits or Lamin A/C. SYBR green qPCR was carried out in triplicate using primers designed to amplify from mRNA of genes targeted, GAPDH and ACTB. Primers were designed and tested as described in Methods. Primers were successfully designed for 12 of the 17 genes tested. Expression levels are shown relative to negative control and were calculated using a variation of the Pfaffl method to allow normalization to multiple housekeeping genes using GAPDH and ACTB to normalize samples (Hellemans et al. 2007). # = genes for which no primers were successfully designed. * no ISYNA1 transcript was detected in cells transfected with siISYNA1.2 (Solid line represents 100% of negative control levels and dashed line represents 30% of control levels). Error bars represent 1 standard error of the mean.

Two possibilities for the observation that some siRNA do not reduce levels of TRAIL-induced Caspase-3/7 activity are that the siRNA does not reduce the levels of the target mRNA sufficiently, or that the target gene is not involved in TRAIL-induced cytotoxicity. To help distinguish between these possibilities levels of mRNA knock-down triggered by siRNA transfection were measured using qRT-PCR (Figure 5.8b). Efficient, specific primers were successfully designed for 12 out of the 17 candidate hit genes. The efficiency of knock-down of the Lamin A/C mRNA by a well characterised siRNA was used as a positive control. Transfection of siLaminA/C reduced levels of the Lamin A/C mRNA to 8.5% of levels in negative control transfected cells, demonstrating the efficiency of the siRNA transfection and qRT-PCR measurement. A total of 20 of the 33 siRNAs designed to target the hit genes tested reduced the level of the targeted mRNA to less than 30% of control levels.

Based on the ability of siRNAs to affect the level of TRAIL-induced Caspase – 3/7 activities and the ability of siRNAs to reduce levels of the intended target mRNA, candidate hit genes were categorised into one of the same four categories defined in the previous chapter. If siRNAs that significantly reduce the level of TRAIL-induced Caspase-3/7 activity are designated phenotypically active siRNAs, and the efficiency of an siRNA is the amount by which an siRNA reduces the intended target mRNA when transfected at a set concentration, then:

- **Confirmed hit genes** are genes targeted by at least two phenotypically active siRNAs that are more efficient than any phenotypically inactive siRNAs targeting the same gene.
- **Unconfirmed hit genes** are genes targeted by only one phenotypically active siRNA, where that siRNA is more efficient than phenotypically inactive siRNAs targeting the same gene, or the efficiency of the siRNAs is unknown.
- **Confirmed off-targets** are genes targeted by both phenotypically active and inactive siRNAs, where at least one phenotypically inactive siRNA is more efficient than the least efficient phenotypically active siRNA.
- **Unrepeatable genes** are genes that are not targeted by any phenotypically active siRNAs.

The results presented in Figure 5.8 along with the categorisations of the candidate hit genes are summarised in Table 5-6. In total six genes were categorised as confirmed hit genes, five as unconfirmed hit genes and five as off-targets. No genes were categorised as unrepeatable. One gene, ISYNA1, could not be categorised, due to the lack of a result for

5. AN siRNA SCREEN OF THE DRUGGABLE GENOME

the efficiency of siRNA 2 targeting ISYNA1. In this case the amplification of ISYNA1 transcript for the transfected cells was below the detection limit of the thermocycler used. This could be due to siRNA 2 targeting ISYNA1 knocking down the ISYNA1 mRNA sufficiently to make it undetectable, or due to a failure in the protocol.

<i>Gene</i>	<i>siRNA</i>	<i>Repeat</i>	<i>Caspase-3/7 significant</i>	<i>Caspase-3/7 Rank</i>	<i>>=70% KD</i>	<i>KD Rank</i>	<i>Conclusion</i>
ACO1	1	+	-	2	+	2	Unconfirmed
	2	+	+	1	+	1	
MAX	1	+	+	2	+	1	Confirmed Hit
	2	+	+	1	+	2	
MAD	1	+	+	1	+	2	Off-Target
	2	+	-	2	+	1	
MYC	1	+	+	1	+	2	Confirmed Hit
	2	+	+	2	+	1	
TEGT	1	+	+	2	+	1	Confirmed Hit
	2	+	+	1	+	2	
RAD9A	1	+	+	2	-	1	Off-Target
	2	N/A	-	3	-	2	
	3	N/A	+	1	-	3	
TLR4	1	+	+	1	N/A	N/A	Unconfirmed
	2	N/A	-	2	N/A	N/A	
	3	N/A	-	3	N/A	N/A	
LRPAP1	1	+	+	1	-	3	Off-target
	2	N/A	-	3	+	1	
	3	N/A	+	2	+	2	
IGF1R	1	+	+	1	-	2	Confirmed Hit
	2	N/A	+	2	-	3	
	3	N/A	+	3	+	1	
HAPIP	1	+	+	1	-	3	Off-Target
	2	N/A	-	2	+	1	
	3	N/A	-	3	+	2	
INADL	1	+	+	1	-	2	Confirmed Hit
	2	N/A	-	3	-	3	
	3	N/A	+	2	-	1	
PDE11A	1	+	+	2	N/A	N/A	Confirmed Hit
	2	N/A	+	3	N/A	N/A	
	3	N/A	+	1	N/A	N/A	
ISYNA1	1	+	+	1	+	2	
	2	N/A	+	2	N/A	1	
	3	N/A	-	3	+	2	
ADORA1	1	+	+	1	N/A	N/A	Unconfirmed
	2	N/A	-	3	N/A	N/A	
	3	N/A	-	2	N/A	N/A	
OR1E2	1	+	+	1	+	1	Unconfirmed
	2	N/A	-	2	-	2	
	3	N/A	-	3	-	3	
GPR132	1	+	+	1	N/A	N/A	Unconfirmed
	2	N/A	-	2	N/A	N/A	
	3	N/A	-	3	N/A	N/A	
CCNT1	1	+	+	1	+	2	Off-Target
	2	N/A	+	2	-	3	
	3	N/A	-	3	+	1	

Table 5-6 Summary of confirmation experiments and categorisation of candidate hit genes. KD: Knock-down
PDE11A was categorised as a “hit” despite the lack of measurements as to the

efficiency of the siRNAs targeting it. Since all three siRNAs targeting PDE11A are phenotypically active, any combination of ranking for the efficiencies would still lead to the categorisation of the gene as a “hit”.

Three genes are categorised as off-target despite being targeted by two independent siRNAs (RAD9A, LRPAP1 and CCNT1). This is due to the genes being targeted by a third, phenotypically inactive, siRNA which is more efficient than either of the other two active siRNAs. This is unexpected, as it is assumed that two siRNAs will not share off-target effects and therefore if two independent siRNAs targeting the same gene have the same phenotype, that the effect is unlikely to be due to off-target effects. This was the case for one gene (PTP4A) from the kinase and phosphatase screen. One possibility is that the assay is sensitive to the non-specific effects of the siRNAs on the cell. If activating the siRNA pathway in general were affecting the assay, then it would be expected that all siRNAs would have the same effect. It is possible that some aspect of the particular siRNAs used here have a differential effect on non-specific responses. Several sequence motifs have been reported to stimulate innate immune responses to siRNAs, such as UGUGU (Judge et al. 2005) and GUCCUCAA (Hornung et al. 2005). Of the siRNAs used for confirmation of hits in this study only siMYC.B contains either of these sequences (UGUGU). Further, generally only immune cells express TLR 7 and 8 which are necessary for the recognition of these sequences (Judge, Maclachlan 2008). However, it is known that poor quality or impure siRNA preparations can induce non-specific responses (Marques et al. 2006). Thus certain siRNAs (or siRNA preparations) maybe affecting the assay independent of the gene knock-down stimulated by them.

In light of this ambiguity, it is safer to designate these genes as potential off-target genes rather than confirmed off target genes. Only two genes (MAD and HAPIP) are therefore designated confirmed off-target.

5.6 Characterisation of hit genes

In order to further investigate the involvement of genes targeted by two phenotypically active siRNAs in the apoptotic pathway, the effect of transfection of these siRNAs on TRAIL-induced Caspase-8 and Caspase-9 was measured using luminescent caspase assays (Figure 5.9). siRNAs targeting the potential off-target genes are included in an attempt to further investigate the nature effects caused by these siRNAs.

Transfection of 10 out of the 20 siRNAs tested significantly reduced the level of TRAIL-induced Caspase-8 activity (Figure 5.9a). Interpretation of the data suffers from the

large variances observed in some cases. Despite this, data from the two siRNAs targeting each gene were in agreement in all but two cases (MAX and ISYNA1). In this case of MAX it is likely that this is due to the large amount of variation seen in the measurement of Caspase-8 activity in TRAIL-treated siMAX.1 transfected cells. There are four cases in which both siRNAs targeting a gene caused a significant reduction in TRAIL-induced Caspase-8 activity (MYC, RAD9A, INADL and LRPAP1).

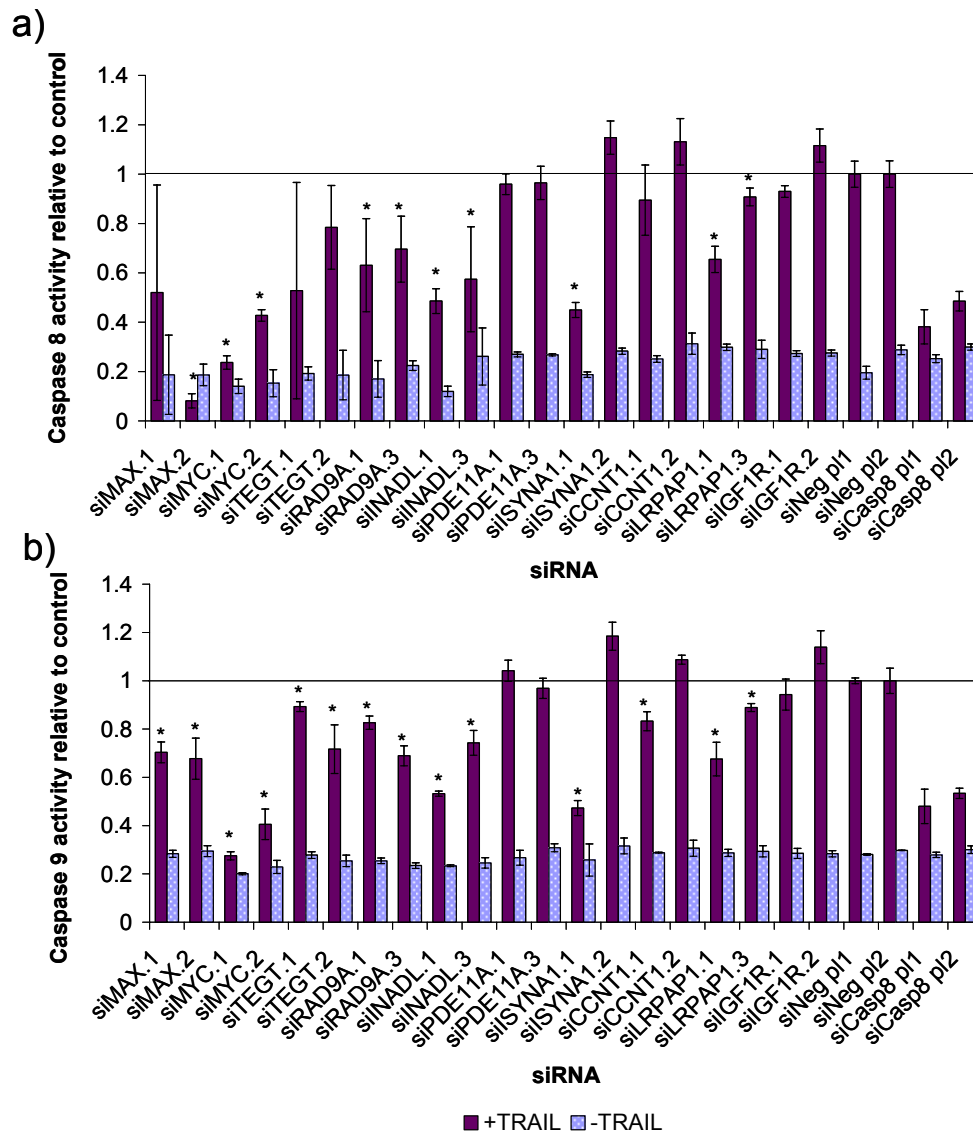


Figure 5.9 Effect of transfection of siRNAs targeting hit genes on TRAIL-induced Caspase activity
 Cells were transfected with siRNAs targeting possible hit genes, siCasp8 or siNeg in sextuplet on two plates. 48 hours post-transfection, cells were treated with either 0.5µg/ml TRAIL or media for 6 hours. Levels of a) Caspase-8 or b) Caspase-9 were measured using Promega Caspase-Glo luminescent caspase assays. Results are expressed relative to the caspase activity levels in TRAIL-treated negative control transfected cells. Horizontal line represents negative control levels. Error bars represent 1 standard deviation, n = 3. * indicates significant difference. siRNA/shRNAmir transfected, the effects should be similar, independent of the siRNA/shRNAmir transfected. Therefore it can be assumed that the differences between different siRNAs/shRNAmirs is the result of the differing effects of that construct on the pay.

Transfection of 14 out of 20 siRNAs significantly reduced the level of TRAIL-

induced Caspase-9 activity (Figure 5.9b). In all but two cases (ISYNA1 and CCNT1) data from the two siRNAs targeting each gene were in agreement. Both siRNAs targeting a gene significantly reduced the level of TRAIL-induced Caspase-9 activity in six out of ten genes (MAX, MYC, TEGT, RAD9A, INADL and LRPAP1). Four of these genes were targeted by 2 siRNAs that induced a significant reduction in TRAIL-induced Caspase-8 activation (MYC, RAD9A, INADL and LRPAP1). In the case of MAX, one siRNA significantly reduced the level of TRAIL-induced Caspase-8 activity, while the other showed a reduction that wasn't statistically significant, possibly due to the high level of variability. One gene (TEGT) was targeted by two siRNAs that significantly reduced levels of Caspase-9, but not Caspase-8 (although a non-significant reduction in TRAIL-induced Caspase-8 activity was observed).

In two cases genes were targeted by siRNAs, transfection of neither of which caused a significant reduction in either Caspase-8 or Caspase-9 (PDE11A and IGF1R). This information is summarised in Table 5-7.

<i>Gene</i>	<i>siRNA</i>	<i>Caspase-8</i>	<i>Caspase-9</i>	<i>Caspase-3</i>	<i>Confirmed?</i>
MAX	1	-	+	+	
	2	+	+	+	+
MYC	1	+	+	+	+
	2	+	+	+	
TEGT	1	-	+	+	+
	2	-	+	+	
RAD9A	1	+	+	+	-
	3	+	+	+	
INADL	1	+	+	+	+
	3	+	+	+	
PDE11A	1	-	-	+	+
	3	-	-	+	
ISYNA1	1	+	+	+	
	2	-	-	+	
CCNT1	1	-	+	+	-
	2	-	-	+	
LRPAP1	1	+	+	+	-
	3	+	+	+	
IGF1R	1	-	-	+	+
	2	-	-	+	

Table 5-7 Summary of effects of transfection of siRNAs from potential hits on TRAIL-induced caspase activity

+ signifies that a significant reduction in activity was observed, - that no significant reduction was observed. Confirmed indicates if gene was classed as a confirmed hit (+) or a potential off-target (-). See Table 5-6.

The evidence presented here suggests that siRNAs targeting MYC, RAD9A, INADL and LRPAP1 act to regulate the apoptosis pathway upstream of Caspase-8. It is also possible that MAX acts upstream of Caspase-8 since the lack of significance in the case of the effect of siMAX.1 on Caspase-8 activity could be due to the large amount of variation observed. Two of these (RAD9A and LRPAP1) were designated as potential off-targets in confirmation experiments (Figure 5.8 and Table 5-6). Despite the fact that both siRNAs

targeting these genes induce the same effect on TRAIL-induced Caspase activity, this does not demonstrate that the effects observed here are not due to the knock-down of off-target genes. While transfection of TEGT did not produce a significant decrease in Caspase-8, this could be due to the large variance. Indeed, no hypothesis test can demonstrate the truth of the null hypothesis – in this case that there is no difference between cells transfected with hit siRNAs and those transfected with the negative control. Therefore, all that can be concluded here is that the involvement on TEGT in regulation of TRAIL-induced Caspase-8 activity has not been demonstrated. Similarly, transfection of both PDE11A and IGF1R failed to produce significant reductions in TRAIL-induced activity of either Caspase-8 or Caspase-9. Variability here was smaller than in the case of TEGT, however, it is possible, from these results, that knock-down of PDE11A or IGF1R cause a small, but real, reduction in the activity of Caspase-8 and -9 activity. Calculating the 95% confidence limit on the mean difference between Caspase-8 activity levels in the negative control transfected cells and in siIGF1R.1/2 transfected cells ($-7\% \pm 9\%$ for siIGF1R.1 and $+12\% \pm 14\%$ for siIGF1R.2) shows that there is a 95% confidence that these siRNAs do not cause a reduction in Caspase-8 levels greater than 16% and 2% (the lower bounds of the confidence intervals) respectively for siIGF1R.1 and siIGF1R.2 compared to the levels in the negative control. The equivalent figures for siPDE11A.1 siPDE11A.2 are 15% and 14% respectively. Confidence limits on Caspase-9 levels are similar (data not shown).

Results from the siRNAs for ISYNA1 and CCNT1 differ for the two siRNAs targeting these genes. CCNT1 was designated a potential off-target gene. These results do not prove that the effects of transfection with siRNAs targeting CCNT1 are off-target effects: the effect of transfection of siCCNT1.2 is weaker than siCCNT1.1 in the Caspase-3 assay (Figure 5.8a) and it is possible that a significant effect is not seen in the Caspase-9 assay due to a lower sensitivity. However, they do make it more likely that the results are due to off target effects. In the case of ISYNA1, siISYNA.1 clearly has a far larger effect than siISYNA.2 in all the caspase assays, arguing against siISYNA.2 being more efficient at knocking down the ISYNA1 transcript and so suggests that ISYNA1 should be categorised as an off-target gene.

5.7 Analysis of seed sequences

From rigorous confirmation experiments on genes targeted by the top 20 scoring siRNAs, six genes have been identified as confirmed hits (MAX, MYC, TEGT, IGF1R, INADL and PDE11A). A further 5 genes were categorised as unconfirmed hits (ACO1,

TLR4, ADORA1, OR1E2 and GPR132). Five genes were categorised as either potential or confirmed off-targets (MAD, RAD9A, LRPAP1, HAPIP and CCNT1). One gene (ISYNA1) could not be categorised, although evidence from experiments designed to determine the point at which targeted genes were acting in the apoptosis pathway suggested ISYNA1 might also be an off-target.

These results suggest that a large number of results seen in the screen might be due to off-target effects. An important factor in the determination of miRNA specificity is the sequence of the so-called “seed region”: this is the region of the miRNA corresponding to positions 2-7 or 2-8 of the mature miRNA sequence or the equivalent region of the target sequence. Several lines of evidence suggest that at least some off-target effects of siRNAs might be due to matches between the seed region of the siRNA and the 3' UTR of transcripts, causing the siRNA to act as a miRNA in repressing these transcripts (Birmingham et al. 2006, Grimson et al. 2007, Lin et al. 2005, Lin et al. 2007, Sætrom et al. 2007). This could lead to the knock-down of one or several unintended transcripts. Nielsen *et al* used the number and length of seed matches between an siRNA and a 3'UTR, along with the AU content and conservation of the surrounding sequences to predict the fold change which the siRNA would induce in the mRNA. In this way they accurately predicted which mRNAs would have the largest change in expression levels following transfection of the siRNA (Nielsen et al. 2007).

In a screen for genes which sensitize normally resistant cells to a Bcl-2/Bcl-XL inhibitor Lin *et al* found that the top three hits were due to off target effects. Further, they found that two of the siRNAs targeting these genes shared a 7nt seed sequence (a heptamer seed, see Figure 1.4 for definition of different types of seed sequence).

To determine if any of the hits in the screen presented here share seed sequences, the seed sequences were extracted and compared from the 40 siRNAs targeting each of the genes to which the top 20 scoring siRNAs from the screen were designed. Seed sequences that appear more than once are shown in Table 5-8. Three hexamer seed sequences (bases 2-7 of the siRNA guide strand or bases 15-20 of the target sequence) appeared more than once in the top 20 siRNAs from the screen, with one sequence appearing in four different siRNAs. In addition one hexamer seed sequence appeared both in an siRNA from the top 20 scoring siRNAs from the screen and also in the second siRNA targeting TEGT, which was later shown in confirmation experiments to induce a significant reduction in TRAIL sensitivity (Figure 5.8a). During confirmation experiments, siRNAs not used in the screen were obtained where only one of the siRNAs in the screen targeting a gene reproducibly gave

a phenotype (e.g. siNADL.3 and siPDE11A.3). These siRNAs were also checked, but in no case did any of these siRNAs contain a seed sequence also found in other siRNAs which targeted candidate hit genes. If only siRNAs which gave a statistically significant result in confirmation experiments are considered, there are three hexamer seed sequences that appear in more than one siRNA in this collection, with one of these appearing in three confirmed siRNAs from the druggable genome screen and one siRNA from the kinase and phosphatase screen.

Only four of the 15 siRNAs targeting confirmed hit genes from the druggable genome screen contained one of these seeds and in all but one case each, of these confirmed hit genes targeted by an siRNA containing one of the seeds was also targeted by an independent siRNA not containing one of the seeds. This suggests that these are still valid hits.

Sampling was used to assess the significance of this observation. 5,000 samples of 20 siRNAs were drawn at random from the list of all siRNAs in the screen. The seed sequences of these siRNAs were determined and compared to determine if any seed sequences appeared multiple times. The probability of any seed appearing more than once in a random sample of 20 siRNAs was found to be 0.1828, the probability of two seed sequences appearing two or more times in a random sample of 20 siRNAs was 0.0128 and the probability of a seed sequence appearing 4 or more times in a random sample of siRNAs was less than 0.0002. This suggests that the observation that two seed sequences appear twice in the top 20 siRNAs from the screen is significant and the observation that one seed sequence appears in four siRNAs from the top 20 is highly significant. Using the same approach to assess the significance of finding the same seed sequences in more than one of the 14 siRNAs from the top twenty scoring siRNAs that were confirmed in confirmation experiments gives p-values of 0.08, 0.003 and less than 0.0002 for finding any seed sequence appearing in at least two siRNAs, finding any two seeds appearing in at least two siRNAs and finding any seed appearing in three or more siRNAs respectively. Two of the seed sequences that appeared multiple times in the top 20 siRNAs from the druggable genome screen also appeared in the siRNAs used to confirm hits from the kinase and phosphatase screen (Table 5-8). In addition two seed sequences that appeared only once in the top 20 siRNAs also appeared in the siRNAs used to confirm hits from the kinase and phosphatase screen.

These results suggest perhaps some of the effect of the ten siRNAs with these seed sequences on TRAIL sensitivity may be due to an off-target or effect or effects shared between siRNAs containing the same seed sequence. It is possible that this may be one of

the confirmed hit genes from the screen. While a match between the seed sequence and the 3' UTR of a transcript is partially predictive of the siRNA having a silencing effect on the transcript, many transcripts with a hexamer seed match will not be silenced. Both Birmingham *et al* and Nielsen *et al* showed that a heptamer seed match, with a match between bases 2 -8 of the siRNA guide strand and the 3' UTR of a transcript has a higher predictive value than a hexamer match, although the sensitivity is reduced (Birmingham *et al.* 2006, Nielsen *et al.* 2007). Further, since there are more possible heptamers than hexamers, a heptamer appearing more than once in the top 20 siRNAs would have an increased significance.

Seed Size	Seed Sequence (target)	Druggable Genome Screen			Kinase Screen		
		siRNA	Rank	Confirmed siRNA Gene	siRNA	Confirmed siRNA Gene	
Hexamers							
	CAAGGT	siTEGT.B	1	+	+		
		siCCNT1.B	3	+	-		
	TGTCCA	siSYNA.B	2	+	+	siINPP5D.2	- -
		siKCNV1.B	17	-	-		
	ACTTGA	siACO1.B	4	+	-	siSharpin.1	+ +
		siHAPIP.B	5	+	-		
		siINADL.B	7	+	+		
		siMAGED.A	14	-	-		
	AGATCA	siGPR132.B	9	+	-	siIKBKE.1	+ +
		siTEGT.A	376	+	+		
	GCATTA	siLOC402037.A	20	-	-	siPPP2CB.2	+ -
Heptamers							
	TCAAGGT	siTEGT.B	1	+	+		
		siCCNT1.B	3	+	-		
	GTGTCCA	siSYNA.B	2	+	+	siINPP5D.2	- -
		siKCNV1.B	17	-	-		
	AACTTGA	siINADL.B	7	+	+		
		siMAGED.A	14	-	-		
	CACTTGA	siACO1.B	4	+	-	siSharpin.1	+ +
	GAGATCA	siGPR132.B	9	+	-		
		siTEGT.A	376	+	+		

Table 5-8 Repeated seed sequences from druggable genome screen results

Table shows seed sequences which appear more than once in siRNAs targeting genes targeted by the top 20 siRNAs from druggable genome screen, or together in one of the top 20 siRNAs from druggable genome screen and in one of the siRNAs targeting candidate hits from kinase and phosphatase screen. Sequences shown are the siRNA target sequences complimentary to bases 2-7 (Hexamers) or bases 2-8 (Heptamers) of the siRNA guide strand. siRNAs labelled A or B are siRNAs from A or B plates of the library. Those numbered 1-3 are siRNAs used for hit confirmation. Rank shows what rank the siRNA was in the screen results. 'Confirmed' shows whether the gene targeted by the siRNA was categorised as a confirmed hit.

Three heptamers appear more than once in the top 20 highest scoring siRNAs from the screen of the druggable genome. One of these appears in two siRNAs that were confirmed. There is one heptamer seed sequence that appears once in the top 20 scoring siRNAs from the screen of the druggable genome and once in the siRNAs used to confirmed hits from the kinase and phosphatase screen (both of which were confirmed), and one heptamer seed that appears once in the top 20 scoring siRNAs from the druggable genome screen and also appears in the second TEGT siRNA, which was shown in subsequent experiments to significantly reduce the sensitivity of cells to TRAIL-induced apoptosis (Figure 5.8 and Table 5-8). The probability of a heptamer seed appearing in a random sample of 20 siRNAs from the druggable genome library is 0.048, while the probability of two and three seeds appearing more than once in a random sample of 20 siRNAs from the library is 0.001 and less than 0.0005 respectively (based on 2,000 random samples of 20 siRNAs from the library of siRNAs used in the screen). The probability of finding a seed appearing in any two siRNAs if only the 14 confirmed siRNAs are considered is 0.03.

The region of an miRNA from base two to base eight is known as the 7mer-m8 seed (Figure 1.4). A different heptamer, shown to be effective at predicting miRNA targets is the 7mer-A1 site, which is the hexamer match flanked by an A in the target at the base that corresponds to base 1 of the siRNA guide strand (Lewis, Burge & Bartel 2005). Since all siRNA in the library have a U at this position, all the siRNAs that share a hexamer match also share a 7mer-A1 seed. Similarly, all those siRNAs that share a 7mer-m8 seed also share an octamer (8mer) seed (bases 1-8 of the siRNA guide strand, Figure 1.4). From here, on the set of seed sequences listed in Table 5-8 are referred to as “hit” seeds (hit hexamers/hit heptamers).

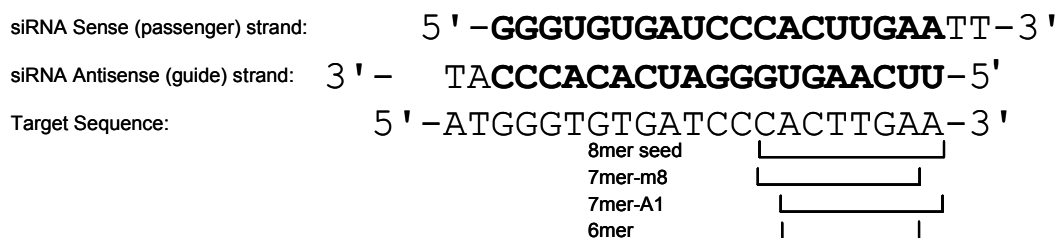


Figure 5.10 Different types of seed sequence as defined by (Lewis, Burge & Bartel 2005)

Seeds are shown in order of the predictive power of a match between the seed sequence of a miRNA and a UTR in predicting transcripts that will be affected by a miRNA.

Within each group of siRNAs from the top 20 scoring siRNAs sharing a common seed sequence, only one siRNAs targets a gene that was confirmed. However there are two cases of siRNAs targeting confirmed hit genes, one from this screen and one from the kinase

Seed	Size	Enrichment Score	Normalized Enrichment Score	Nominal P-value	FWER p-val	Rank at Max	Leading Edge
Hexamers							
TAATAA	70	0.542	2.691	<0.001	0	1942	44%
ACTTGA	14	0.846	2.674	<0.001	0	946	79%
CCTTAA	16	0.793	2.642	<0.001	0	1367	81%
AATTAA	44	0.566	2.567	<0.001	0	1798	45%
ACTGGA	10	0.877	2.510	<0.001	0	933	90%
TAGGAA	13	0.786	2.505	<0.001	0	621	54%
TAAAGA	20	0.685	2.482	<0.001	0.002	1659	45%
GAATAA	21	0.716	2.474	<0.001	0.002	1902	57%
AAGTTA	20	0.651	2.426	<0.001	0.004	1963	60%
TCACAA	12	0.792	2.413	<0.001	0.005	1723	75%
AAATGA	21	0.662	2.405	<0.001	0.005	2030	52%
AGATCA	35	0.588	2.378	<0.001	0.006	1728	60%
TTATAA	22	0.650	2.335	<0.001	0.009	1978	50%
AATATT	15	0.698	2.314	<0.001	0.015	3088	80%
AGATCT	17	0.666	2.304	<0.001	0.015	2808	71%
TGAATA	16	0.673	2.255	<0.001	0.026	2085	81%
CTGGAA	8	0.848	2.198	<0.001	0.047	341	50%
Heptamers							
CAATTAA	18	0.817	2.911	<0.001	0.000	1154	67%
GAGATCA	18	0.696	2.387	<0.001	0.001	600	50%
GAAAGAA	10	0.790	2.241	0.005	0.015	2173	90%
TTAATAA	17	0.624	2.227	<0.001	0.015	1555	41%
GTATTTA	16	0.671	2.211	<0.001	0.016	2928	81%
ATAGGAA	6	0.923	2.187	<0.001	0.021	467	67%
ACCTTAA	8	0.828	2.185	<0.001	0.021	1284	88%
TAATTAA	8	0.812	2.179	<0.001	0.024	1798	63%
TTAATTA	10	0.776	2.151	<0.001	0.037	2464	100%
ACAATTA	10	0.753	2.148	<0.001	0.039	2069	80%
AAGATCA	13	0.659	2.140	0.007	0.041	1643	69%
CTAATAA	20	0.595	2.139	<0.001	0.041	1043	45%
CTAATTA	7	0.855	2.136	<0.001	0.043	1042	71%

Table 5-9 Seed sequences enriched in high scoring siRNAs

Table shows results of applying GSEA to the ranked list of siRNAs from the druggable genome screen using “gene sets” composed of siRNA sequences which share a hexamer or heptamer seed. The size column refers to the size of the set (i.e. the number of siRNAs containing that seed), (normalized) enrichment score is the statistic calculated by GSEA. Rank at max is the position in the ranked list of siRNAs that the enrichment score is maximal, and the Leading edge is the proportion of genes in the gene set that rank at or higher than this point.

and phosphatase, sharing a common seed sequence.

Increasing the length of the seed increases the predictive power of that seed in determining the identity of transcripts regulated by those siRNAs sharing this seed.

However, it reduces the sensitivity of the process. As such, although siRNAs which share a heptamer seeds are more likely to share some of the same off-target effects, using the hexamer seed increases the chance of finding siRNAs that share off-target effects.

5.7.1 Gene Set Enrichment Analysis of seed sequences

The process of RNAi screening is designed to enrich for siRNAs that produce a phenotype of interest. As such, it can be assumed that the screening process will enrich not only for siRNAs designed to target genes involved in the process of study, but also siRNAs which target genes involved in the process through ‘off-target’ effects (Lin et al. 2007). In the previous section, seed sequences were found multiple times in siRNAs targeting the genes targeted by the top 20 siRNAs. However, defining the top 20 siRNAs as a cut off is arbitrary. In order to determine if any seeds had been enriched generally in high scoring siRNAs, gene set enrichment analysis (GSEA, see 0) was applied to the results of the druggable genome screen. The hexamer and heptamer seed sequence of every siRNA used in the screen was determined and ‘gene sets’ were constructed where every set was composed of all siRNAs from the screen that shared a particular seed sequence. GSEA was then applied to the ranked list of all siRNAs from the screen using these gene sets in order to identify sets of siRNAs, all containing the same seed, which were enriched at the top end of the ranked list of siRNAs. A FWER significance cut-off of 0.05 was applied to determine which sets were enriched in the high-scoring siRNAs. The output from this analysis of ‘gene sets’ based on both hexamer and heptamer seeds is shown in Table 5-9. The analysis shows that 17 hexamer and 13 heptamer seeds are enriched in the high-scoring siRNAs, (defining a set of seed sequences, referred to from here on as “enriched” seeds. These are distinct from, but overlapping with, the “hit” seeds). Two of the enriched hexamers and one of the enriched heptamers are among the seed sequences in Table 5-8. Three of the enriched hexamer seeds and one of the enriched heptamer seeds which are not among the hit seeds are found in top 20 siRNAs: The hexamer CCTTAA is found in siMYC.A, TAAAGA is found in the siRNA siMAD.A and the hexamer/heptamer (C)TAATAA is found in the siRNA siADORA.B

The enrichment of these seed sequences in the high scoring siRNAs shows siRNAs containing these seed sequence have an increased probability of inducing the phenotype of interest when transfected. There are two possible explanations for this. Firstly it is possible that these seed sequences are highly efficient sequences, and that siRNAs containing these sequences are, therefore, more likely to knock-down the intended target sufficiently to induce the phenotype of interest. The second possibility is that the enriched seed sequences specify

siRNAs containing them to knock-down off-target transcripts, some of which could be among the hits from the screen, which are involved in the process of interest. That is, as predicted by Lin *et al*, the screening process itself is enriching for off-target effects. However, it is important to note that in no case is the “rank at max” close to the very top of the list, nor in most cases is the proportion of siRNAs containing this seed which appear at or above the “rank at max” 100%. This suggests that the score of an siRNA in the screen is not purely determined by its seed sequence.

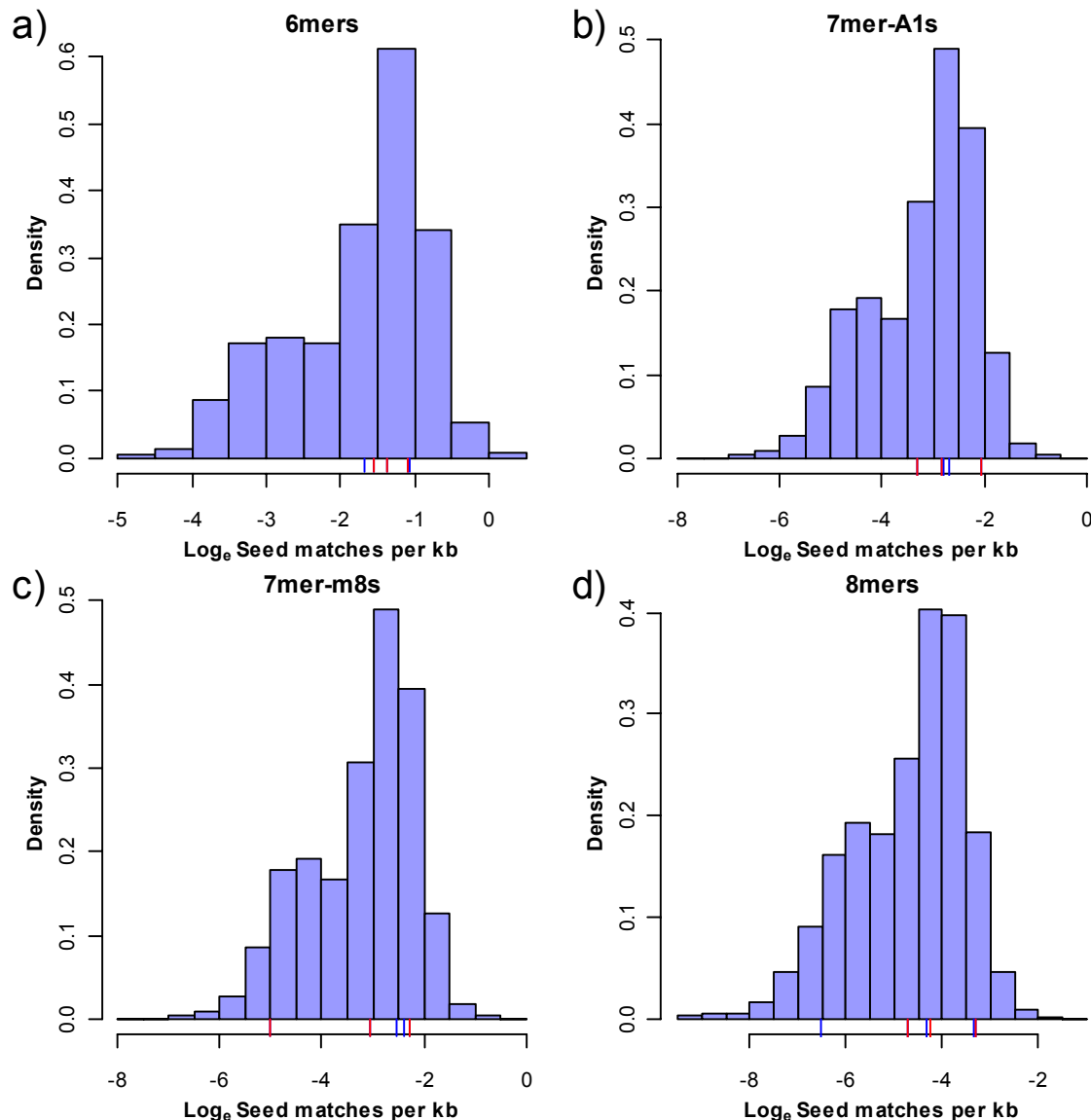


Figure 5.11 Average frequency of ‘Hit’ seed sequences in all 3’ UTRs compared to average frequency of all possible seed sequences

3’UTR sequences were obtained from ensembl (release 46). Each 3’ UTR was searched for matches to every possible 6nt (a), 7nt (b/c) and 8nt (d) sequence. Frequency of each sequence per kb of each UTR was calculated and averaged (using a script written by Dr. A. Enright). The distributions of \log average sequence frequency were plotted. The average frequency of each of the a) hit 6mer seeds, b) hit 7mer-A1seeds, c) hit 7mer-m8 seeds and d) hit 8mer seeds is shown as a blue tick beneath the histograms, seeds present in two or more confirmed siRNAs are shown in red.

5.7.2 Frequency of hit and enriched seed sequences in 3' UTR sequences

siRNAs containing seed sequences that are found more frequently in the 3'UTR of transcripts are likely to have a larger number of off-target effects. Further, finding multiple matches between a 3' UTR and an siRNA seed sequence has a higher predictive value for off targets than the presence of a single match (Birmingham et al. 2006). Thus it is the total frequency of seed matches in a 3' UTR that is important, rather than just the presence or absence of such matches.

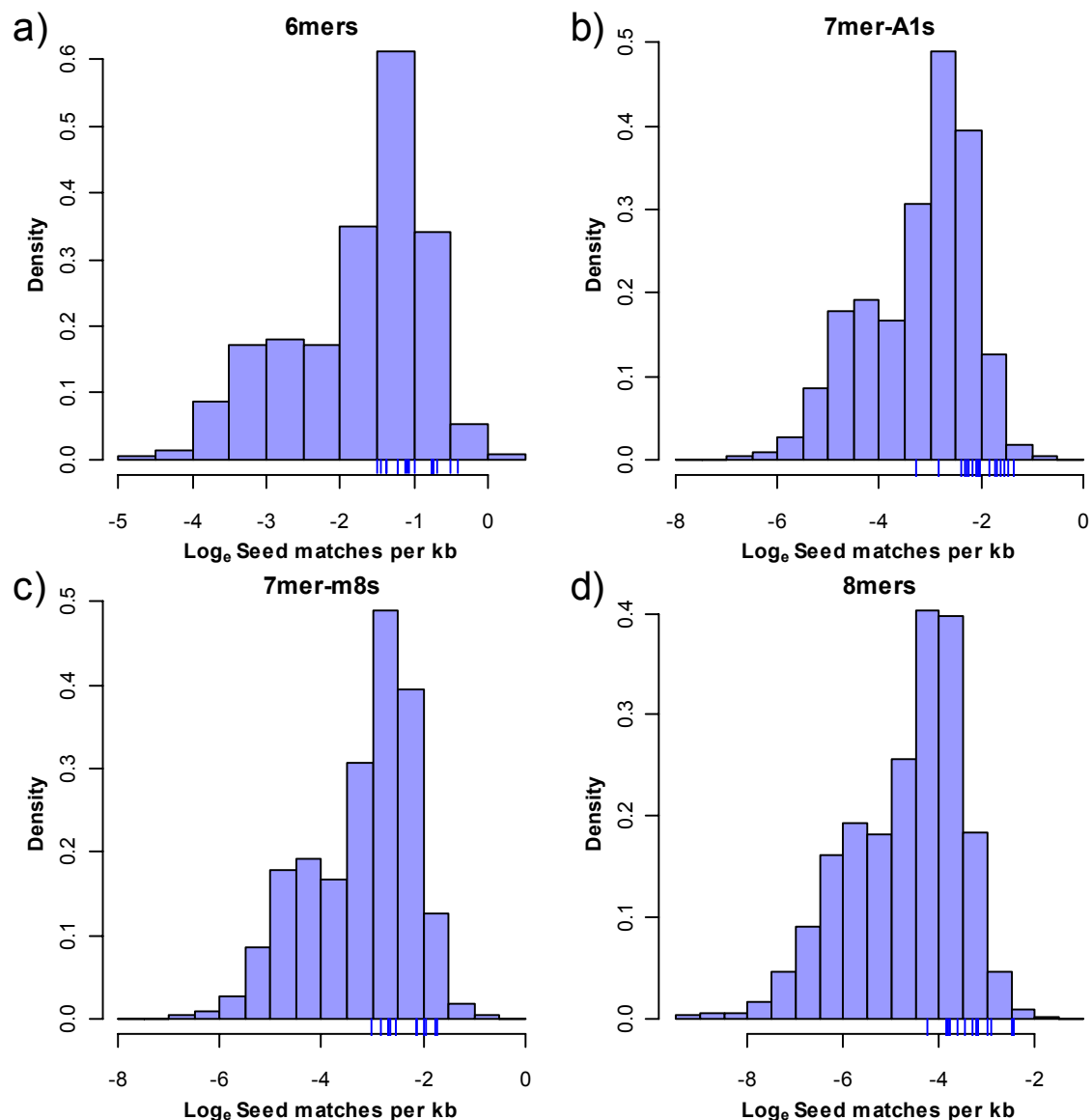


Figure 5.12 Frequencies of ‘enriched’ seed sequences in all 3' UTRs compared to the all possible seed sequences.

Distributions of frequencies of all possible sequences were calculated as described for Figure 5.11. The frequencies of a) enriched hexamers seeds (6mers), b) enriched 7mer-A1 heptamer seeds, c) enriched 7mer-m8 heptamer seeds and d) enriched octamer (8mer) seeds are indicated by tick marks under the histograms.

To examine the possibility that the hit/enriched seed sequences are found at an unusually high frequency in the 3' UTRs of transcripts, the average frequency of every possible six nucleotide, seven nucleotide and eight nucleotide sequence in each of 3' UTRs contained in the complete set of ensembl 3' UTRs was calculated (using a script written by Dr. A. Enright) and their distributions plotted (Figure 5.11). The average frequency of each of the hit hexamer seeds (Figure 5.11a), both the 7mer-A1 and 7mer-m8 hit heptamer seeds (Figure 5.11b and Figure 5.11c) and the hit octamer seeds (Figure 5.11d) were marked on the plots.

The distributions of average log seed match frequencies for all possible seeds form bimodal normal distributions, with a large peak on the right-hand, higher-frequency end of the plot and a smaller peak on the left-hand, lower frequency end of the plot. The significance of the minor peak is unclear. One possibility is that the sequences in this peak have some biological function and are therefore selected against by evolution (e.g. they are miRNA targets). Another alternative is that the sequences in the minor peak could contain a different base composition to that generally found in 3' UTRs. The hit seeds all fall towards the higher end of the total range of frequencies. However, hit seed sequences are mostly located towards the centre of the major peak. That is, if those sequences which are unusually under-represented are not considered, the hit seed sequences have neither an unusually high or low frequency in 3' UTRs compared to average frequencies of all possible seed sequences. An exception to this is the heptamer/octamer sequence GCGATCA(A), which is clearly in the part of the distribution containing under-represented sequences.

The frequencies of the enriched seed sequences were plotted on the same distributions (Figure 5.12). Unlike the hit seed sequences, the enriched seed sequences cluster towards the higher end of the major peak. This effect becomes stronger as the length of the sequence increases. This suggests that those seed sequences that are enriched in the high scoring siRNAs have a larger number of off-target effects, increasing the chance that they will knock-down a larger number of genes connected to the TRAIL-induced cytotoxicity pathway.

5.7.3 Occurrence of hit seed sequences in genes previously associated with the TRAIL pathway

It is possible that siRNAs which cause a large change in TRAIL-induced cytotoxicity and contain a hit seed may be exerting their effect in part by reducing the levels of genes demonstrated to be associated with the TRAIL pathway either here, or previously, (it is likely

that part of the effect may still be due to reduction of the intended target).

Seed Sequence	Gene hit at least once	Genes hit at least twice
6mers		
CAAGGT*	INADL, TEGT, IRAK1, PRKAA2, FBXO11, MAPK10	INADL
TGTCCA	INADL, DIABLO , BID , IRAK1, FADD	INADL
ACTTGA*	INADL, IGF1R, BID , TNFRSF10B , PRKAA2, TNFRSF10A , PRKCD, PRKCQ, FBXO11	INADL, TNFRSF10B , TNFRSF10A , PRKCQ, FBXO11
AGATCA*	INADL, TEGT, TNFRSF10B , PRKAA2, PRKCD, PRKCQ, FBXO11	INADL, TNFRSF10B , PRKAA2
GCATTA	INADL, TNFRSF10B , PRKAA2, IKBKE, TNFRSF10A , WDFY4, FBXO11	TNFRSF10B , PRKAA2, IKBKE, TNFRSF10A , FBXO11
7mer-A1		
CAAGGTA*	INADL, TEGT	
TGTCCAA	IRAK1	
ACTTGAA*	BID , PRKAA2, TNFRSF10A , PRKCD, PRKCQ, FBXO11	TNFRSF10A , FBXO11
AGATCAA*	PRKCQ	
GCATTAA	TNFRSF10B , PRKAA2, TNFRSF10A , WDFY4, FBXO11	
7mer-m8		
TCAAGGT*	INADL, TEGT	
GTGTCCA	IRAK1, FADD	
AACTTGA	TNFRSF10B , TNFRSF10A , PRKCQ	
CACTTGA*	INADL, IGF1R, TNFRSF10B , TNFRSF10A	INADL, TNFRSF10A
GAGATCA*		
8mer		
TCAAGGTA*	TEGT	
GTGTCCAA	IRAK1	
AACTTGAA	TNFRSF10A	
CACTTGAA*	TNFRSF10A	
GAGATCAA*		

Table 5-10 Genes associated with the TRAIL pathway with 3' UTR matches to "hit" seeds

Human 3' UTR sequences were retrieved from ensembl 46. The UTRs of genes previously associated with the TRAIL pathway, or confirmed hits from the two screens presented here, were searched for matches to seed sequences which were either repeated in the siRNAs targeting the top 20 siRNAs, or appeared in both these siRNAs and the siRNAs used to confirm the hits from the kinase and phosphatase screen. Genes in bold are 'core' TRAIL genes, which were not associated with the TRAIL pathway through RNAi screening. *Seeds found in two or more confirmed siRNAs.

In order to discover if siRNAs which cause a large change in TRAIL-induced cytotoxicity and contain hit seed sequences may be reducing the level of genes previously

associated with the TRAIL pathway, the 3' UTRs of these genes were searched for matches to the hit seed sequences. Genes shown to be involved in TRAIL mediated apoptosis, either previously or in this work, with either one or two matches to hit seed sequences in their 3'UTR are shown in Table 5-10. All of the hit hexamer seed sequences are found twice in the UTRs of at least one of the genes associated with the TRAIL pathway, other than the one the siRNA containing the seed is designed to target. All of the hit 7mer-A1 and 4 of the 5 hit 7mer-m8 seeds are found in at least one of the UTRs of genes associated with the TRAIL pathway, other than the one the siRNA containing the seed is designed to target. For each type of heptamer, one seed is found twice in the UTR of two of these genes. Similarly, matches to all but one of the 8mer seeds are found in the UTR of at least one of these genes. In order to assess the significance of this, the 3' UTRs of these genes were searched with all the seed sequences appearing in the library, and the percentage of seeds that were found either once or twice in at least one of these genes recorded (Table 5-11). More than 75% of hexamer or either of the types of heptamer seed are found one or more times in the 3'UTR of at least one of the TRAIL genes. Large numbers of seed sequences are also found twice at least one of the TRAIL pathway genes (72% for 6mer seeds, 24% for 7mer-A1 seeds and 22.7% for 7mer-m8) seeds.

The gene set used above includes genes associated with the TRAIL pathway through the use of RNAi screening. Removing these genes produces a smaller set of genes (first column, Table 1-2), the 'core' genes. Genes from this 'core' set, which contain hit seed sequences are highlighted in Table 5-10, and the percent of all seed sequences from the library found in the 3' UTRs of one or more of these genes is shown in Table 5-11. A large number of seed sequence are found in the 3' UTRs of 'core' TRAIL genes, with 80% of hexamer seeds, 39% of 7mer-A1 seeds and 43% of 7mer-m8 being found in at least one.

Seed Type	At least one match per UTR		At least two matches per UTR	
	<i>All genes</i>	<i>Core genes</i>	<i>All genes</i>	<i>Core genes</i>
6mer	94.7%	80.1%	72.7%	31%
7mer-A1	76.8%	39.3%	23.9%	4.25%
7mer-m8	80.0%	42.5%	22.7%	4.45%
8mer	41.6%	13.5%	4.05%	0.476%

Table 5-11 Percentage of screen seeds found in the 3' UTR TRAIL genes

3' UTRs of genes associated with TRAIL-induced apoptosis, either here or previously (all genes) or genes with a well established role in TRAIL-induced apoptosis (Core genes, see Table 1-2) were searched for matches to all the seeds found in siRNAs used in the druggable genome screen.

This finding suggests that either a large number of the siRNAs used in the screen are knocking-down genes previously associated with the TRAIL pathway, or that the seed matches are not sufficient to specify off-target effects. Under either of these hypotheses the finding that high scoring siRNAs contain seeds which are found in the 3' UTR of genes

involved with the TRAIL pathway is not significant, as many other siRNAs in the library that did not score so highly also have seed sequences which are found in these 3' UTRs. However many of the hit seed sequences are found in the 3' UTR of not just one, but several genes associated with the TRAIL pathway. One explanation for the overrepresentation of these seed sequences in high scoring siRNAs could be that in part their effect on TRAIL sensitivity is due to the additive effect of small reductions in levels of transcripts for a number of genes involved in the TRAIL pathway.

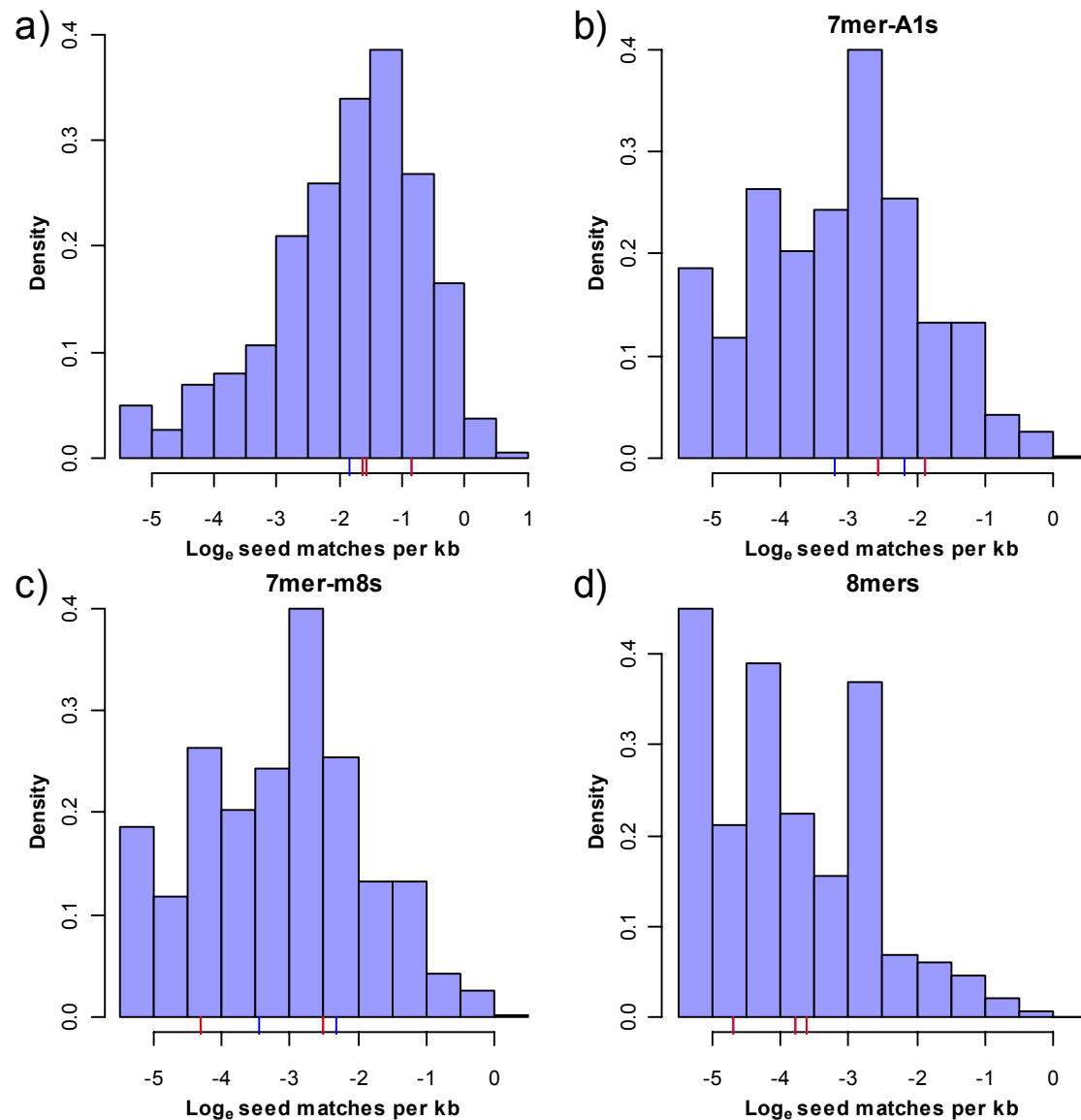


Figure 5.13 Average frequencies of “hit” seed sequences compared to all possible seed sequences in 3' UTRs of genes previously associated with the TRAIL pathway

Average frequencies of all possible seed sequences calculated as described for Figure 5.11, except using 3' UTRs associated with the TRAIL pathway, either in this work or previously. Frequency of a) hit hexamers seeds(6mers), b) hit 7mer-A1 heptamer seeds, c) hit 7mer-m8 heptamer seeds or d) octamer (8mer) hit seeds are shown by tick marks under the histograms. Seeds found in two or more confirmed siRNAs are shown in red.

If siRNAs containing the hit seeds are causing a reduction in the level of transcripts

previously associated with the TRAIL pathway, then the average frequency of hit seed sequences in each of the 3' UTRs of these genes should be higher than other possible seed sequences of the same length.

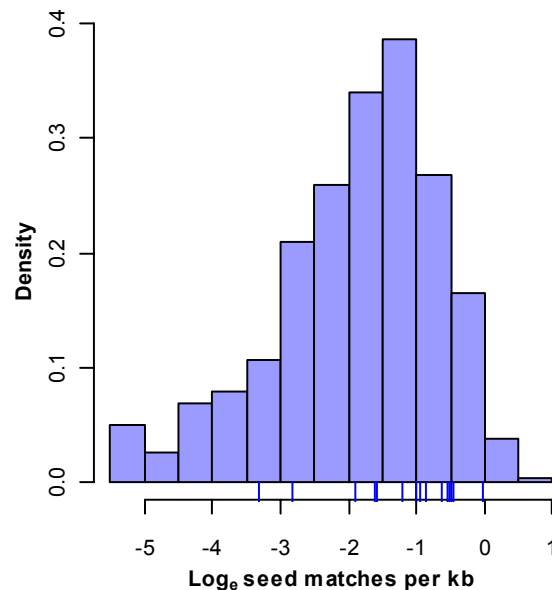


Figure 5.14 Frequency of “enriched” hexamer seeds compared to all possible hexamer seeds in 3’UTRs of genes previously associated with the TRAIL pathway

Average frequency of all possible hexamer seeds were calculated as for Figure 5.. Tick marks show frequency of “enriched” seed sequences

The distribution of average frequencies of all possible 6nt (Figure 5.a), 7nt (Figure 5.b/Figure 5.c) and 8nt (Figure 5.d) sequences in each of the 3' UTRs of genes associated with the TRAIL pathway either here or in previous work was calculated. The average frequencies of hit seed sequences were examined relative to these distributions. In all cases the average frequencies of the hit seed sequences were contained within the central portion of the distribution of all possible sequences. This was also the case when using only “core” genes (data not shown). This shows that the 3' UTRs of genes previously associated with the TRAIL pathway are not enriched for matches to the hit seed sequences, and therefore does not support the idea that siRNAs containing these seed sequences are more likely to knock-down genes previously associated with the TRAIL pathway than siRNAs containing other seed sequences. Examining the average frequencies of enriched seeds gives a similar result, with the possible exception of the enriched hexamers, which do cluster slightly to the right of the main peak, although the effect is weak (Figure 5.14, data not shown).

5.7.4 Identifying possible off-target transcripts for hit and enriched seeds

If the seed region of an siRNA is important in determining the specificity of the siRNA with regards to off-target effects, then, by searching for matches to seeds in 3' UTR

sequences, it may be possible to identify novel candidate transcripts that are unintended targets of these siRNAs. Lin *et al* used the two heptamer seed sequences that occurred in their top three hits to search transcripts that might be responsible for the effect observed (Lin *et al.* 2007). They found that 3,312 and 2,503 genes contained each of the heptamer seeds. To further reduce the number of candidates they looked at the overlap between these sets. The overlapping set contained 343 genes, including Mcl-1, a member of the Bcl-2 family. Bcl-2 proteins were known to be involved in the process being studied. It was confirmed that the siRNAs containing these seeds did knock-down Mcl-2, and new siRNAs targeting Mcl-2 scored well in their assay.

Ensembl human 3' UTR sequences (www.ensembl.org, release 46) were searched for matches to hit and enriched seed sequences. Matches to at least one of the five hit hexamers were found in 9,523 UTRs and matches to at least one of the 17 enriched hexamers were found in 14,827 UTRs (around 50% of the genes in the genome). There are 171 UTRs that contain matches to all 5 hit hexamer seeds and 54 that contain hits to all 17 enriched hexamers. These lists of hit can be reduced in several ways. The first is to require that each UTR contains two matches to each seed. There are 10 UTRs which contain two matches to all of the 'hit' hexamer seed sequences and 3 UTRs which contain two matches to all 17 of the enriched hexamer seeds. The second method for reducing the number of possible candidates is to use heptamers (either the hexamer with a flanking A – the 7mer-A1 site or the 7mer-m8 seed) or octamers (7mer-m8 with a flanking A) in the search. There are no UTRs which contain matches to all hit or enriched 7mer-A1 seeds or the 8mer seeds. There is one UTR hit by all of the hit 7mer-m8 seeds (the UTR of CCDC93) and two hit by all of the enriched 7mer-m8 seeds (the UTRs of FZD3 and THAP2).

Longer 3' UTRs can be expected to contain more matches to a given set of seed sequences than shorter sequences by chance. In order to correct for this, the total number of matches to any seed within one of the seed sets (hit hexamers/enriched 7mer-A1 heptamer seeds etc.) was normalised to the length of the UTR in kilobases. For each seed type UTRs were ranked according firstly to the number of independent hit or enriched seed sequences contained within the UTR and then by the frequency of matches to any of the hit or enriched seed sequences (Table 5-12 and Table 5-14). Table 5-13 presents the same analysis for seeds found in at least two confirmed siRNAs. These lists provide candidates for genes which may be involved in regulation of sensitivity to TRAIL-induced cytotoxicity but that were missed in the primary screen. For example, the 3' UTR of the gene AKAP11 contains

Rank	6mers			7mer-A1s			7mer-m8s			8mers		
	Gene Symbol	Seeds Matched	Match Frequency	Gene Symbol	Seeds Matched	Match Frequency	Gene Symbol	Seeds Matched	Match Frequency	Gene Symbol	Seeds Matched	Match Frequency
1	AKAP11	5	5.87	PPP3R2	5	1.79	CCDC93	5	1.05	Q9HCM6	3	1.22
2	NP_849153	5	5.02	AKAP11	4	3.67	CES3	4	2.38	IPO9	3	0.36
3	INADL	5	4.89	AGPAT5	4	2.20	CD300E	4	1.81	C3orf1	2	3.87
4	RNUXA	5	4.79	EDG2	4	1.85	TPMT	4	1.70	C10orf137	2	3.00
5	AGPAT5	5	4.40	ETS1	4	1.67	ZBTB43	4	1.25	Q86TT0	2	2.78
6	CRNKL1	5	4.38	KLK2	4	1.59	Q6ZT99	4	1.18	SLC25A20	2	2.57
7	KLK2	5	3.97	NP_001004299	4	1.56	CD59	4	0.98	MOSC1	2	2.06
8	NP_001009555	5	3.88	SOX1	4	1.39	HIVEP3	4	0.98	IRF2	2	2.01
9	CTTNBP2NL	5	3.73	ZNRF3	4	1.29	ATRN	4	0.94	Q4G197	2	1.96
10	EDG2	5	3.71	C1orf151	4	1.27	AKAP12	4	0.90	KCNV1	2	1.85
11	ATE1	5	3.69	FNDC3B	4	1.24	WHSC1	4	0.88	LOH12CR1	2	1.77
12	VAV3	5	3.68	TPD52	4	1.23	SEC31B	4	0.85	TNFRSF10A	2	1.75
13	CDV3	5	3.61	NP_001032309	4	1.19	IPO9	4	0.85	PNPLA3	2	1.69
14	XK	5	3.54	ZNF275	4	1.17	CXorf39	4	0.82	TOP1	2	1.68
15	SLC6A20	5	3.40	BRWD1	4	1.16	KCTD12	4	0.80	ARF1	2	1.67
16	Q6ZTR4	5	3.35	NP_001017980	4	1.16	MIB1	4	0.64	LEF1	2	1.65
17	ETS1	5	3.34	ZKSCAN1	4	1.11	AFF2	4	0.54	CPOX	2	1.62
18	MTRF1L	5	3.31	SPATA17	4	1.07	TNRC6B	4	0.40	NP_001013646	2	1.52
19	LDLRAD3	5	3.29	ALS2CR13	4	1.00	EIF2C3	3	3.96	TMEM155	2	1.47
20	PPP3R2	5	3.22	CD47	4	0.95	AKAP11	3	2.94	AKAP11	2	1.47

Table 5-12 Top 20 3' UTRs containing matches to "hit" seed sequences

Ensembl 3'UTRs were searched for matches to hit seed sequences. Both the number of independent seeds which matched the 3' UTR (seeds matched) and the total number of matches to any of the hit seeds were calculated. The total number of matches to any of the hit seeds was normalised to the length of the UTR (match frequency, in matches per kb). Shown are the top 20 3'UTRs ranked first by number of seeds matched and then by the match frequency.

Rank	6mers			7mer-A1s			7mer-m8s			8mers		
	Gene Symbol	Seeds Matched	Match Frequency	Gene Symbol	Seeds Match	Match Frequency	Gene Symbol	Seeds Match	Match Frequency	Gene Symbol	Seed Matches	Match Frequency
1	NP_612420.1	3	17.44	CROT	3	2.61	EIF2C3	3	3.96	AKAP11	3	2.20
2	KRBA2	3	8.35	HISPPD2A	3	2.48	K0415	3	0.78	LCP2	2	3.27
3	ZNF701	3	7.47	AKAP11	3	2.20	CCDC93	3	0.63	TMEM156	2	3.22
4	NOB1	3	6.52	AGPAT5	3	1.76	GSN	2	5.62	Q86TT0	2	2.78
5	POLR1E	3	6.06	RUNDC1	3	1.52	GPR34	2	4.05	CMBL	2	1.70
6	ZNF761	3	5.95	EDG2	3	1.39	ARPC2	2	3.58	ZNF696	2	1.68
7	C10orf58	3	5.57	O94914	3	1.22	RYK	2	2.94	HISPPD2A	2	1.65
8	ZNF600	3	5.11	KLK2	3	1.19	GJB6	2	2.74	KIAA0753	2	1.41
9	DBF4B	3	4.95	COL5A1	3	1.18	C18orf19	2	2.71	Q8N849	2	1.41
10	IL12RB2	3	4.93	FLJ43980	3	1.17	LONRF2	2	2.65	TSR1	2	1.34
11	SNX21	3	4.77	THOC5	3	1.16	FAM29A	2	2.25	GDF8	2	1.29
12	RPIA	3	4.67	CTSB	3	1.15	AKAP11	2	2.20	CINP	2	1.22
13	ZNF397	3	4.65	PPP1R15B	3	1.11	TCF7L1	2	2.18	ELAVL4	2	1.15
14	Q8N1I6	3	4.46	PPP3R2	3	1.07	C9orf57	2	2.17	SUV39H2	2	1.11
15	CMKLR1	3	4.43	C6orf107	3	0.99	ANGPTL7	2	2.09	DAP	2	1.10
16	Q6ZR34	3	4.38	C1orf151	3	0.96	MGC12966	2	2.08	FAM98B	2	0.95
17	PIPOX	3	4.38	FNDC3B	3	0.93	BTN3A3	2	1.99	PARP11	2	0.92
18	Q8N9A9	3	4.38	Q6ZSF1	3	0.92	ZNF784	2	1.98	EDG7	2	0.89
19	MRP63	3	4.35	PLCB1	3	0.91	Q8N1N1	2	1.96	SLC26A4	2	0.85
20	RASGEF1B	3	4.27	FLJ27459	3	0.89	C1orf167	2	1.93	TCF12	2	0.84

Table 5-13 Top 20 3' UTRs containing matches to "hit" seed sequences found in two or more confirmed siRNAs.

Ensembl 3'UTRs were searched for matches to hit seed sequences that are found in two or more confirmed siRNAs. Both the number of independent seeds which matched the 3' UTR (seeds matched) and the total number of matches to any of the hit seeds were calculated. The total number of matches to any of the hit seeds was normalised to the length of the UTR (match frequency, in matches per kb). Shown are the top 20 3'UTRs ranked first by number of seeds matched and then by the match frequency.

Rank	6mers			7mer-A1s			7mer-m8s			8mers		
	Gene Symbol	Seeds Matched	Match Frequency	Gene Symbol	Seeds Matched	Match Frequency	Gene Symbol	Seeds Matched	Match Frequency	Gene Symbol	Seeds Matched	Match Frequency
1	NR1D2	17	15.48	GABRA4	16	3.65	THAP2	13	5.38	THAP2	11	3.97
2	DCDC2	17	14.12	CPEB4	15	6.00	FZD3	13	3.11	FZD3	9	1.60
3	ARHGAP5	17	14.02	CXorf23	15	5.62	MAN1A1	12	5.36	TMEM106B	8	1.70
4	CENTB2	17	12.89	FZD3	15	5.60	LRRC8C	12	3.96	RAB22A	8	1.41
5	C1orf96	17	12.80	ARL5B	15	4.98	ACOT11	12	3.59	ZNRF2	7	4.76
6	ATP11B	17	12.76	CKAP5	15	3.88	CRSP2	11	4.86	EDG2	7	3.71
7	CXorf23	17	12.60	OGT	15	3.35	SLC30A1	11	4.45	SLAIN2	7	2.52
8	GABRA4	17	12.23	SLC1A2	15	3.30	TFEC	11	4.08	PPP3R2	7	2.51
9	FZD3	17	11.37	ACVR2A	14	6.76	SLC4A7	11	3.96	TMEM48	7	2.50
10	CPEB3	17	11.20	FLRT3	14	6.23	FSD1L	11	3.53	SLC4A7	7	2.23
11	LGALS8	17	10.79	MOBKL1A	14	6.05	REEP3	11	3.53	RAB11FIP2	7	2.21
12	NP_689969	17	10.72	LGALS8	14	5.19	NP_001017980	11	3.48	TPBG	7	1.95
13	PDE7B	17	10.72	CPD	14	5.17	EXOC5	11	3.43	ZBTB41	7	1.94
14	VAPA	17	10.63	TMEM106B	14	5.11	MIB1	11	3.34	EXT1	7	1.91
15	PGM2L1	17	10.57	PGM2L1	14	4.73	SYNCRIP	11	3.30	SOCS4	7	1.76
16	RBM12	17	10.56	PLCXD3	14	4.33	NP_872329	11	3.28	LRRIQ2	7	1.63
17	PDE10A	17	10.28	NHLRC2	14	4.27	CRB1	11	3.09	CRB1	7	1.63
18	GRIN3A	17	10.21	CRB1	14	4.07	VAPA	11	2.61	PLCXD3	7	1.55
19	ANGPT2	17	9.86	BRWD1	14	3.99	OGT	11	1.93	ARL5B	7	1.40
20	LANCL3	17	9.85	TLOC1	14	3.95	ENAH	11	1.92	KIAA2022	7	1.37

Table 5-14 Top 20 3' UTRs containing matches to “enriched” seed sequences

Number of matches and match frequency for each of the types of seed sequence was calculated as for Table 5-12 except using enriched seed sequences rather than hit seed sequences.

matches to all of the hit hexamer seeds, four of the five 7mer-A1 heptamer seeds, three of the five hit7mer-m8 heptamer seeds and two of the five hit octamer seed sequences. In each case, the frequency of these matches within the 3' UTR of AKAP11 is high enough to place the gene in the top twenty for each of the seed types (Table 5-12). AKAP11 is an A-kinase anchor protein. Such proteins are involved in controlling the localization of protein kinase A. AKAP11 has been shown to form a complex with protein kinase A and GSK3 β , thereby allowing protein kinase A to regulate the activity of GSK3 β (Tanji et al. 2002). Regulation of GSK3 β has been shown to be important for the regulation of TRAIL sensitivity in MYC over-expressing cells (Rottmann et al. 2005). Given the number of genes that have been associated with TRAIL-induced apoptosis, it is fairly likely that any collection of genes of the size of the collection in Table 5-12 there will be related to TRAIL-induced apoptosis in this sort of way. Interestingly, however, the 3' UTR of INADL is also found in the list of top 20 3'UTRs containing matches to the hit hexamers. INADL is one of the genes selected for confirmation from the screen and was confirmed in the confirmation experiments (Table 5-2 and Table 5-6).

These lists of possible effectors of the off-targets effects contain too many genes with a zero score to be able to test for enrichment of gene sets using GSEA. While it is not possible to test for enrichment of previously identified TRAIL pathway genes in high-scoring genes, it is possible to test for enrichment of these genes within genes meeting a certain significance criteria using a chi-squared test. The lists of genes containing a match to one or more of the hit or enriched seed sequences within their 3' UTRs were tested for enrichment of genes previously associated with the TRAIL-induced apoptosis pathway. The lists of genes containing a match to one or more of the hit hexamer or hit 7mer-A1 heptamers have a significant enrichment of genes previously associated with the TRAIL-induced apoptosis pathway ($p=0.044$ and $p=0.0201$). When only seeds that appear in one or more of the confirmed siRNAs are considered, only the enrichment of 7mer-A1 seeds remains significant ($p=0.0343$), but this could be due a reduction in the sample size of genes containing a match to the seed sequences. The list of genes whose 3' UTRs contain a match to one or more of enriched hexamers has a highly significant enrichment of genes previously associated with TRAIL-induced apoptosis ($p=0.0016$). Lists of genes whose 3' UTRs contain matches to other seed types (e.g. enriched heptamers, hit octamers etc) were not significantly enriched in genes previously associated with TRAIL-induced apoptosis at the 5% level.

5.7.5 Micro RNA seeds

mirBase is a database of miRNA sequences. In order to determine if any of the hit or enriched seeds from the screen are shared with natural miRNA sequences, the sequences of all human miRNAs were obtained from mirBase and searched for matches to the hit and enriched seeds from the screen. Four of the seeds from the screen are also found in natural miRNAs (Table 5-15). It is interesting to note that ACTTGA is the seed sequence of the human miRNA miR-26a, as this seed sequence appears four times in the top 20 siRNAs, and is the seed which scored second highest in the analysis of seed enrichment. ACTTGA is also found 9 times in the 3'UTR of DR5 (TNFRSF10B), 4 times in the 3' UTR of DR4 (TNFRSF10A) and 3 times in the 3'UTR of BID, all important genes in the TRAIL-induced apoptosis pathway. Counting the number of ACTTGA sites per kilobase of UTR in genes previously associated with TRAIL-induced apoptosis and in all 3'UTRs as a whole (which is not the same as the average frequency with which it appears in each 3'UTRs as calculated above) reveals an enrichment for this seed sequence in the 3' UTRs of genes previously associated with the TRAIL pathway. The frequency of the seed sequence ACTTGA is three times higher in UTR sequence from genes associated with the TRAIL pathway than it is in all UTR sequence. This could suggest that miR-26a could be involved in controlling the sensitivity of the cell to TRAIL.

<i>Seed</i>	<i>miRNA</i>
ACTTGA	has-miR-26a
GCATTA	has-miR-155
ACTGGA	has-miR-145
TAGGAA	has-miR-384

Table 5-15 “Hit” and “enriched” seed sequences also found in natural human miRNAs

5.8 Discussion and conclusions

A screen of siRNAs targeting genes in the druggable genome was conducted in order to identify genes which affected the sensitivity of cells to TRAIL-induced apoptosis. Genes targeted by the 20 highest scoring siRNAs were selected for confirmation. An initial re-screen of these 20 genes eliminated 3 genes for which neither siRNA from the library caused a significant reduction in sensitivity to TRAIL-induced cytotoxicity. The 17 remaining genes were examined in more careful confirmation experiments. Of the 17 genes, six were categorised as confirmed hits, five as unconfirmed hits, and five as ‘off-targets’. In one case a categorisation could not be given, but later experiments suggested that this was also an off-target gene. Genes for which two siRNAs had a significant effect on TRAIL-induced caspase-3 activity were tested for their ability to affect the TRAIL-induced activity of other

caspases.

The seed sequences targeting potential hits, both from this screen and from the kinase and phosphatase screen, were examined. It was found that five hexamer and five heptamer (7mer-m8) seeds were found more than once in this set of siRNAs (hit seeds). A further 15 hexamer and 12 heptamer (7mer-m8) seeds were found to be enriched in high scoring siRNAs by GSEA (enriched seeds).

5.8.1 The Screen

Before analysis of the screening data was conducted, the relationship between pre-treatment viability and post-treatment survival was examined. A link between cell density and TRAIL sensitivity was observed previously in assay development experiments. It was observed that there was a link between pre-treatment viability and post-treatment survival in the data from the screen. While removing some of the data from the analysis might mean the loss of potentially interesting siRNAs that reduce both cell viability and TRAIL sensitivity, the relationship observed is likely to interfere with analysis further down the line. One of the consequences of removing such siRNAs from the screening data is normalised survivals for many siRNAs based on only available for one replicate. It was decided that in these cases the score for the siRNA should be based on this remaining data point.

A strong relationship was observed between the mean normalized survival of cells transfected with an siRNA and the standard deviation of survival between the two replicates. This relationship was also observed for the data from the kinase and phosphatase screen. However, unlike the data from the kinase and phosphatase screen, log transformation of the data in this screen showed a weaker (although clearly present) relationship. Thus the data from this screen was log transformed during the analysis procedure. The difference between this and the previous screen could simply be one of size, with the reduction in apparent relationship more clear here due to the increased number of data points.

Analysis of the positive control wells in the previous screen showed that there was a drop both in the dynamic range between siCasp8 and siNeg controls, and the Z' -factor between siCasp8 transfected and siNeg transfected wells when compared to assay development experiments. In order to try and reverse this reduction, the screen presented here was conducted using a higher concentration of TRAIL ligand (0.25 μ g/ml in the kinase and phosphatase screen and 0.5 μ g/ml in this screen). However, even with this increase in the concentration of the TRAIL ligand and also the quality control threshold used here (all plates with a dynamic range of less than 2 were repeated, albeit only once) the dynamic ranges of

plates used in the analysis of this screen followed a similar distribution to those from the kinase and phosphatase screen, and further, the Z' –factors between siNeg transfected cells and positive control transfected cells were lower than for the kinase and phosphatase screen (Z' factor for siCasp8, siBID and siSMAC were -0.95, -3.44 and -7.52 respectively here, compared with -0.35, -1.05 and -4.08 for the kinase and phosphatase screen). This suggests that the decrease in relative effect for the positive controls is due to the increase in throughput rather than the concentration of TRAIL. This effect could well be due to the increase in variation when throughput increases, possibly connected with the use of larger numbers of independent cell batches.

In many ways the results from this screen were similar to the results for the kinase and phosphatase screen. The correlations between replicates and between siRNAs targeting the same gene were similar for this screen to those from the previous screen, with the correlation between replicates ($r = 0.57$) being higher than the correlation between siRNAs targeting the same gene ($r = 0.075$). This again suggests that the screening process is more reliable for identifying siRNAs with an effect on TRAIL-induced apoptosis than for selecting genes involved in the process. This was reflected in the findings of the re-screen of siRNAs targeting genes targeted by the top 20 scoring siRNAs from the screen. 71% of siRNAs which had scored highly in the screen gave a statistically significant reduction in TRAIL sensitivity when re-tested in triplicate. In contrast only 38% of second siRNAs targeting these same genes gave a statistically significant reduction in TRAIL-induced cytotoxicity (or conversely 62% of these siRNAs recapitulated the lack of large effect seen in the screen). Six of the genes initially selected for verification by virtue of being targeted by siRNAs in the top 20 were eventually confirmed to be hits (using the definition of a confirmed hit given above). This is 30% of the genes targeted by the 20 top scoring siRNAs, exactly the same proportion of genes selected the same way for confirmation from the kinase and phosphatase screen that were eventually designated confirmed hits. It should, however, be noted that the criteria for confirmation here was slightly higher as siRNAs had to show a significant effect on TRAIL-induced Caspase-3 activity before they were classified as hits. This would suggest that the druggable genome screen has the same accuracy as the kinase and phosphatase screen.

Defining a threshold score for the designation of a hit based on the scores of positive controls proved to be of limited use in the analysis of the data from the kinase and phosphatase screen. Further, if genes affect the sensitivity of cells to TRAIL in a quantitative rather than qualitative way, which would seem at least plausible given that siRNAs affect

TRAIL sensitivity in a quantitative way, defining a binary boundary between hit and non-hits makes little sense. This presents a problem for defining the sensitivity of a screen in terms of the number of genes previously associated with the TRAIL pathway that are identified in the screen as hits. In order to provide some sort of measure of the success of the screen in identifying genes previously associated with the TRAIL pathway, Gene Set Enrichment Analysis (GSEA) was applied to the screening results. GSEA assesses the enrichment of a set or sets of genes within the high scoring portion of a ranked list of genes. The set of genes previously associated with the TRAIL pathway was enriched in the high scoring siRNAs, but with a low level of significance ($p = 0.087$). The enrichment score found was due to the effects of siRNAs targeting Caspase-8, BID, DR4 and MYC: genes which would be expected to have large effects on the sensitivity of cells to TRAIL-induced apoptosis. A similar analysis of the data from the kinase and phosphatase screen failed to identify an enrichment of genes previously associated with the TRAIL pathway in the high scoring siRNAs. This can at least partly be explained by the observation that the genes previously associated with the TRAIL pathway that are targeted by siRNAs in the kinase and phosphatase screen are mainly un-confirmed genes from the Aza-blanc *et al* screen, while the druggable genome screen contained siRNAs targeting well established genes in the TRAIL pathway, knock-down of which is known to have a large effect on TRAIL sensitivity. This identifies a further problem with the use of previously associated genes in assessing the sensitivity of a screen: the quality of the set of genes previously associated.

Controls demonstrate that in the screen, siRNAs targeting genes with known larger effects score more highly than genes known to have a smaller effect on TRAIL-induced apoptosis. However, it is impossible from the data presented to tell if efficiency of this screen in terms of whether the selection of genes using the screening data is better than selecting genes at random. Assuming that the results of the screen were no better than random, this would suggest that 30% of all genes included in the screen were involved in the TRAIL-induced apoptosis pathway. This is not so improbable as might be initially assumed. In a network model of cellular signalling, many or most genes have some effect on the network output. This could be tested by selecting a number of genes at random from the list of genes included in the screen and applying the same confirmation process to them as was applied to the candidate hits selected.

Two genes were identified where the effects of one of the siRNAs targeting them could be shown to be due to off-target effects. One of these was MAD, the binding partner of one of the confirmed hits MAX. MAD competes with MYC for MAX binding and

antagonises the effects of MYC (Luscher 2001). Worryingly, confirmation experiments identified three genes where the effects of siRNAs targeting them on TRAIL sensitivity could be ascribed to off-target effects, despite the fact that two independent siRNAs targeting each gene caused a significant reduction in TRAIL-induced cytotoxicity, as a third siRNA targeting each gene reduced the transcript levels further than either of the other two, without causing a reduction in TRAIL-induced cytotoxicity. Current best practice for RNAi experiments suggests that phenotypes should be confirmed by the use of at least two siRNAs targeting the same gene although some in the field argue for the use of three siRNAs (Echeverri et al. 2006). This finding supports the use of at least three siRNAs in confirming a phenotype, particularly when one of the siRNAs is from a screen, since screening can be shown to enrich for off-target effects (see below). This finding suggests that those confirmed hits which are targeted only by both library siRNAs (MYC, MAX and TEGT from this screen and Sharpin from the kinase and phosphatase screen) require further confirmation. Indeed, both siRNAs targeting TEGT are among the set of “hit” seeds that appear multiple times in the siRNAs which target genes selected for confirmation, suggesting that it is possible that the effect of both siRNAs targeting TEGT is due, at least in part, to off-target effects. This raises the question of how many siRNAs are necessary to confirm a hit. In theory it is possible even for genes targeted by three siRNAs to be shown to be off-targets due to a fourth siRNA not giving a phenotype. Conversely the finding that a third siRNA does not induce a phenotype because it is not as efficient at silencing the targeted transcript does not add any evidence either way. This suggests that real confirmation of the involvement of a gene in a process must come from a rescue experiment, where a non-silenceable form of the target gene is reintroduced into the cell, or, alternatively the involvement of the gene is confirmed using some other, non-RNAi, technique such as a small molecule inhibitor.

The increase in variability seen in the results of this screen compared to the smaller screen and assay development experiment clearly presents difficulties in the interpretation of data. Part of variance may be attributable to differences between the increased number of batches of cells used. It is possible that this could be decreased by sub-cloning the cells used in the experiment. This would allow the selection of a clone with a consistently lower level of surviving cells in the negative controls. If no such clone of HeLa cells were found, it may have been beneficial to test other TRAIL sensitive cell lines in the assay. Further, the power of the screen may have been increased by optimising the assay using a less powerful positive control. For example 2.5pmol of siCasp8 was sufficient to see an almost complete abrogation

of TRAIL sensitivity. However, high quantities may have been required to reliably distinguish siRNAs with a smaller effect on the sensitivity of cells from negatives. In both these screens the dynamic range between positive and negative controls was used to assess the quality of each plate. However, dynamic range does not account for the variance seen in the controls, only the average value. The Z'-factor is a better measure of the distance between two distributions, but relies on measures of variance which are not meaningful with only two replicates. In future it may be beneficial to include more replicates of positive (and possibly negative) controls on each plate. In order to make room for this, it would be necessary to include fewer controls in total. Finally, it is pertinent to return to the question of whether it would have been beneficial to perform the screen in triplicate rather than duplicate. While for an individual datum point, taking the minima of two points is more conservative, on average than taking the mean of three, it is not clear that this is the case when considering the ranking of all genes. Further, it could be argued that one of the issues with the screen is that the results were overly conservative – that is there was a high number of false negatives – arguing that a less conservative approach may have been beneficial. However, performing a third replicate on the druggable genome would have involved the expenditure of significantly more resources, leaving fewer resources available for confirmation and follow up work. One possibility would have been to perform three replicates, but not repeat poor-quality plates. It is clear that a third high-quality replicate in the kinase screen would have allowed the question of whether the use of the extra resources is justified, given the extra expense, to be addressed.

5.8.2 Hit and enriched seeds

The seed sequences of the top siRNAs targeting the genes targeted by the top 20 siRNAs and the siRNAs targeting candidate hits from the kinase and phosphatase screen were examined to look for sequences that appeared more than once. Five hexamer seeds appeared more than once in this set and one appears in four separate siRNAs. Three of these five sequences are found in two or more siRNAs that showed a statistically significant effect on TRAIL sensitivity in confirmation experiments. Five heptamer (7mer-m8, see Figure 1.4) seeds also appeared more than once in this set of siRNAs (3 if only confirmed siRNAs are considered). Together these seed sequences make up the set of “hit” seed sequences (Table 5-8). GSEA was used to look for seed sequences that were enriched in siRNAs that scored highly in the screen. This analysis identified 17 hexamer and 13 heptamer seed sequences where siRNAs containing these seeds were enriched in the high-scoring siRNAs. These seed

sequences define the set of “enriched” seeds. Two of the hit hexamer seed sequences and one of the hit heptamer seed sequences were also in the set of enriched seeds (Table 5-9). Because all of the siRNA guide strands in the library start with a U at the 5’ end, all siRNAs that share a hexamer seed also share a 7mer-A1 heptamer seed and all siRNAs that share a 7mer-m8 heptamer seed also share an octamer (8mer) seed (Figure 1.4). These were generally found in siRNAs targeting genes categorised as unconfirmed or off-target.

This suggests that as hypothesised by Lin *et al*, the process of screening enriches for siRNAs with off-target effects which affect the process being studied (Lin et al. 2007). This is not unexpected, since screening selects for siRNAs which have a phenotypic effect on the assay. However, for the purpose of the assay there is no difference between an off-target and an on target effect. Therefore, if the library contains siRNAs which off-target genes involved in the process, then these siRNAs will inevitably be enriched in the top scoring siRNAs for the screen. If off-target effects are specified by the seed sequence of the siRNA, then since the library contains siRNAs contain around 2000 different hexamer seeds out of 4096 possible hexamer seeds, it is likely that multiple siRNAs which off-target any gene will be found in the library. It is important to note that not all siRNAs containing these sequences score highly in the screen, this suggests either the seed sequence is not the sole determinant of off-target specificity, that the off-target effects are mostly weak, or likely both.

As the length of a seed match increases its positive predictive power increases but the sensitivity of using it to predict off-target effects decreases. For example Birmingham *et al* took 84 mRNAs that were significantly down regulated by an siRNA and 84 which were not. They found that 84% of the down regulated transcripts contained a match to the siRNA hexamer seed, while 17% of the negative set had a match. These numbers were 69% and 8% respectively for heptamer matches (Birmingham et al. 2006). A similar effect is seen with increasing numbers of matches. Nielsen *et al* find that the effect of the number of matches is multiplicative and that a single octamar match has the same effect as two heptamer matches (Nielsen et al. 2007).

In order to examine what is causing siRNAs containing hit or enriched seeds to score highly in the screen, the average frequency at which these seed sequences appear in each of the 3’ UTRs for all the human genes in ensembl was calculated and compared to the distribution of the average frequency of all possible seed sequences (Figure 5.11 and Figure 5.12). Hit seed sequences clustered in the central part of the main peak of the distribution of the average frequencies of all possible seed sequences. This suggests that hit seed sequences do not cause a larger number of off-target effects than would be expected by chance. In

contrast, enriched seeds tended to cluster towards the upper part of the frequency distribution of all sequences. This suggests that siRNAs containing these sequences may cause the off-target reduction of a higher number of transcripts than could be expected by chance. Such promiscuous siRNAs are likely to score highly in any screen as it is likely that the sum total effects of knocking down a large number of genes, even by a small amount, is likely to affect many processes. It suggests that siRNA design algorithms which do not already do so should take account of the frequency of seed sequences in 3' UTRs.

At least two matches to the 3' UTRs of at least one genes associated with the TRAIL pathway, either here or previously, were found for all of the hexamers. At least one match to the 3' UTR of at least one of these genes was found for all the 7mer-A1 heptamer seeds, four of the five 7mer-m8 seeds and four of the five octamer seeds (Table 5-10). Surprising however, a very high proportion of all seed sequences were found once in the 3' UTR of at least one of the genes associated with TRAIL sensitivity (94.7% of hexamers, 76.8% of 7mer-A1 heptamers, 80.0% of 7mer-m8 heptamers and 41.6% of octamers). These numbers remained high even when considering the percentage of all seed sequences that match twice in the 3' UTR of one of these genes, or if a more restrictive, higher confidence set of TRAIL genes was used (Table 5-11). These numbers are for seeds found in at least one of the 3' UTRs, while matches to the hit seeds are found in the 3' UTR of several of these genes. It was also found that the average frequency of matches of hit seed sequences to the 3' UTRs of genes associated with TRAIL-induced apoptosis was not any higher than seen for other seed sequences. Thus the siRNAs containing hit sequences did not score highly because they targeted a higher number of the genes previously associated with TRAIL sensitivity than other seed sequences. Performing the same analysis for enriched seeds showed that in general this also held true for the enriched heptamer and octamer seeds. However, the average frequencies of enriched hexamer seeds were clustered in a position slightly to the higher end of the distribution suggesting that these siRNAs may be hitting a larger number of genes associated with the TRAIL pathway than other seed sequences. This is not to say that the 3' UTRs of genes associated with the TRAIL pathway are unusual, rather, that there is a high likelihood of finding a match to any seed sequence in any collection of this many 3' UTRs. Indeed the mean frequency of all hexamers in 3'UTRs is 0.24 matches per kb, meaning a match to any hexamer would be expected in one in every four 1 kb 3' UTRs (Figure 5.11)

Four of the seeds from the hit and enriched seeds are also found in natural miRNAs. The seeds GCATTA and ACTGGA are the hexamer seed of miR-155 and miR-145

respectively. Deregulation of the expression of these miRNAs has been shown in B-cell lymphomas and colorectal neoplasia respectively (Eis et al. 2005, Michael et al. 2003). The seed ACTTGA is the seed sequence of miR-26a. The sequence ACTTGA is found in four of the top 20 scoring siRNAs, one of the siRNAs from the kinase and phosphatase screen and is also the second most enriched seed in high scoring siRNAs. This seed is found nine times in the 3'UTR of DR5, four times in the 3'UTR of DR4 and three times in the 3' UTR of BID. Indeed, per kb of 3' UTR sequence associated with TRAIL genes this seed is three times more frequent than found in total 3' UTR sequence. The frequency of a seed per kb of 3' UTR is subtly different from the average frequency in each 3' UTR. In the situation where a smaller number of UTRs have very high frequencies of seed matches, the former will be higher than the latter, as the latter averages out values from a small number of highly enriched UTRs. Repeating the analyses in 5.7.2 and 5.7.3 using total frequency over all 3' UTRs gives the same results as for average frequency in each 3' UTR except for this seed (data not shown). This suggests that possibly miR-26a is involved in control of sensitivity to TRAIL-induced cytotoxicity. Experiments to determine if siRNAs contain this seed do affect the level of DR4, DR5 and BID transcript / protein will show if this is indeed a possible explanation for the effect of these siRNAs. Experiments to examine the correlation between miR-26a expression and TRAIL sensitivity may also throw light on a possible role for this miRNA in controlling sensitivity to TRAIL-induced cytotoxicity.

Thus it seems likely that siRNAs containing enriched seeds score highly due to a larger number of weak off-target effects rather than particularly targeting genes involved in TRAIL-induced apoptosis. That is the screening process is enriching for genes with a large number off-target effect, rather than specifically enriching for siRNAs with off-target effects on genes involved in TRAIL-induced cytotoxicity. In contrast siRNAs containing the seed sequences ACTTGA may score highly through stronger off-target effects against core genes in the TRAIL pathway as well as the through effect on the intended target. The reason for the high score of siRNAs containing the other "hit" sequences remains unknown.

Despite this evidence that part of the effect of siRNAs containing these sequences may be attributable to off-target effects, several of the genes targeted by these siRNAs were confirmed through the action of independent siRNAs that do not contain over-represented seed sequences. One explanation for this maybe that while part of the effect elicited by the siRNA in question maybe due to off-target effects, part of the effect is also due to knock-down of the intended target. In support of this hypothesis, in all cases where a gene is targeted by two phenotypically active siRNAs, one of which contains an over-represented

seed, the siRNA with the over-represented seed causes a stronger phenotype, irrespective of the relative efficiency of the siRNAs in knocking-down the transcript of the targeted gene. The one hit for which this is not the case is TEGT. In this case both siRNAs targeting this gene contain hit seeds.

It may be possible to use the hit/enriched seed sequences to identify novel genes in the TRAIL pathway. Lin *et al* used the seed sequences from siRNAs scoring highly by off-target effects to identify Mcl-1 as a regulator of sensitivity to Bcl-2/Bcl-XL inhibitor ABT-737 (Lin et al. 2007). Transcripts with matches to heptamer/octamer or multiple hexamer seeds are possible off-targets for an siRNA containing that seed. However, each siRNA will have many off-target effects, only a small number of which may be involved in TRAIL-induced apoptosis. The chance of a gene being involved in the TRAIL pathway is increased by finding matches to multiple different hit/enriched seed sequences. There are many transcripts whose 3' UTRs contain matches to all of the hit hexamers or to all the enriched hexamers. These numbers can be reduced by requiring multiple hits per 3' UTR, which increases the probability that a transcript is affected by siRNAs containing the seed. Therefore transcripts were ranked first by the number of independent hit (Table 5-12) or enriched (Table 5-14) seeds with matches in the 3' UTR and then by the frequency of these matches as ranking purely by match frequency means that the highest ranking genes are ones with very short 3' UTRs containing one seed match. This examination produces a large number of candidate genes. Further investigation will involve devising some form of measure of the significance of finding matches to multiple seeds in a 3' UTR, possibly similar to the method proposed by Nielsen *et al* (Nielsen et al. 2007) to give a smaller number of these genes which can be examined by experimental investigation. One such gene may be AKAP11 which has previously been shown to be involved in the regulation of MYC levels, which known to be involved in the regulation of TRAIL sensitivity.

5.8.3 The Hits

Six genes selected for confirmation from the screening results were categorised as hits by the definition above. That is they are targeted by at least two phenotypically active siRNAs, which are more efficient at reducing the level of the targeted transcript than any siRNA tested which targets the same transcript but was not found to be phenotypically active. In one case – MYC - one of these two siRNAs contained a seed sequence which was enriched in high scoring siRNAs. In a second case – TEGT – both of the siRNAs targeting this gene contained suspect seed sequences.

5.8.3.1 MYC and MAX

The MYC protein (also known as c-MYC) is a multifunction transcription factor, and a prototypical proto-oncogene. It has many roles connected with tumorigenesis including increased proliferation and regulation of both intrinsic and extrinsic apoptotic pathways and is up-regulated in many human cancer types (Reviewed: Nilsson, Cleveland 2003). It is well established that MYC is involved in sensitivity to TRAIL-induced apoptosis (Wang et al. 2004/5, Ricci et al. 2004, Rottmann et al. 2005, Wang et al. 2005). MYC suppresses the transcription of the cFLIP apoptosis inhibitor (Ricci et al. 2004), promotes the transcription of DR5 (Wang et al. 2004/5). It has also recently been shown that MYC can inhibit the pro-survival functions of the NF- κ B subunit RelA which is itself activated by TRAIL signalling (Ricci et al. 2007). Finally, a new model hypothesizes that MYC is involved in the “priming” of the mitochondrial pathway, thereby prompting this pathway to amplify the pro-apoptotic TRAIL signals (Nieminen, Partanen & Klefstrom 2007).

MAX is MYC’s dimerization partner, and is required for both the transcriptional activating and suppressing functions of MYC (Reviewed: Luscher 2001). It is therefore unsurprising that it was also isolated from the screen.

Both siRNAs targeting both MAX and MYC caused a significant reduction in TRAIL-induced Caspase-3/7 activity (a condition of being classified a hit) and TRAIL-induced Caspase-9 activity. Both of the MYC and one of MAX siRNAs caused a significant reduction in Caspase-8 activity. The failure of one of the MAX siRNAs to cause a reduction could be due the large variation observed in the experiment (Figure 5.8a and Figure 5.9). The size of the effect of MYC knock-down on TRAIL-induced Caspase-8 activity suggests that the effect is direct – i.e. it is not the product of a feedback activation of Caspase-8 by other Caspases. This suggests that MYC does have effects on the TRAIL pathway at points other than reducing the inhibition, or priming, of the mitochondrial apoptosis pathway (Luscher 2001, Ricci et al. 2007).

5.8.3.2 IGF1R

The IGF1R (Insulin-Like Growth Factor Receptor) protein is a tyrosine kinase that regulates a number of pathways connected to cancer cell survival and proliferation (Reviewed: Tao et al. 2007). It has been shown that IGF1R signalling activates both Ras/Raf/ERK and AKT signalling pathways, both of which have been shown to be involved in the control of sensitivity to TRAIL (Chen et al. 2001, Frese et al. 2003, Nesterov et al. 2004, Thakkar et al. 2001, Wang et al. 2005). Indeed it was recently shown that

treatment of colon carcinoma cells with the ligand for IGF1R – IGF1 – increased the sensitivity of these cells to TRAIL-induced apoptosis (but protected against TNF α -induced apoptosis). This effect was dependent on the AKT pathway, but not the Ras activated ERK or p38 MAPK pathways and could be enhanced by blocking NF- κ B (Remacle-Bonnet et al. 2005). This is surprising since previous reports have shown that activation of the AKT protects rather than sensitizes cells to TRAIL-induced apoptosis (Chen et al. 2001, Thakkar et al. 2001).

Here three siRNAs targeting IGF1R showed a significant reduction in TRAIL-induced Caspase-3/7 activity (Figure 5.8a). The finding that this effect was induced by all three siRNAs targeting IGF1R, in addition to the fact that none of these siRNAs contain any of the hit or enriched seed sequences adds confidence to the conclusion that the expression of IGF1R is involved in the sensitivity of cells to TRAIL-induced apoptosis. It is interesting to note that knock-down of IGF1R did not have such a large effect on Caspase-8 and Caspase-9 activity (Figure 5.9) as previous reports of the effect of AKT on TRAIL sensitivity have suggested that it acts to control BID cleavage (Chen et al. 2001, Thakkar et al. 2001), although it is possible that a small effect on Caspase-9 has an amplified effect on Caspase-3/7 activity levels. These results support a role for IGF1R/AKT in positively regulating Caspase-3 activation in TRAIL-treated cells in concurrence with the results of Remacle-Bonnet *et al.*

5.8.3.3 PDE11A

As with siRNAs targeting IGF1R, all three siRNAs targeting PDE11A caused a significant reduction in the level of TRAIL-induced Caspase-3/7 activity (Figure 5.8a). None of the siRNAs targeting PDE11A contained any of the hit or enriched seeds, thus allowing a high level of confidence in this gene. Again like IGF1R, knock down of PDE11A did not cause a significant reduction in the levels of TRAIL-induced Caspase-8 or Caspase-9 activity (Figure 5.9).

The PDE11A gene encodes for a dual specificity phosphodiesterase protein (Fawcett et al. 2000) and is widely expressed in many normal tissues and some carcinoma cell types (D'Andrea et al. 2005). Phosphodiesterases are involved in the linearization of cyclic AMP and GMP molecules which are important secondary messenger molecules in cellular signalling. PDE11A is unusual in that it hydrolyses both cAMP and cGMP (Fawcett et al. 2000). cAMP levels in the cell are important as they regulate the activity of Protein Kinase A and the cAMP response element binding (CREB) transcription factor which are both

activated by high levels of cAMP, and are therefore repressed by the action of phosphodiesterases. The gene for the anti-apoptotic protein Bcl-2 contains cAMP response element (CRE) in its promoter region and it has been shown that AKT induces expression of Bcl-2 through the action of the CREB transcription factor on this promoter. Since CREB activity is dependent on cAMP its activity could be reduced through the activity of PDE11A. This would lead to a reduction in the levels of Bcl-2 transcription and thus a reduction in the protection Bcl-2 provides against apoptosis (Pugazhenthii et al. 2000). It was noted above that AKT has been shown to have both pro- and anti-apoptotic roles (Chen et al. 2001, Remacle-Bonnet et al. 2005, Thakkar et al. 2001). Reducing PDE11A expression could serve to alter the balance in away from pro-apoptotic signals.

5.8.3.4 INADL

Two of three siRNAs targeting INADL caused a significant reduction in TRAIL-induced Caspase-3/7 activity. A third siRNA did not significantly alter TRAIL-induced Caspase-3/7 activity (Figure 5.8), however cells transfected with this siRNA had a higher level of INADL expression than cells transfected with either of the other siRNAs targeting INADL. siINADL.1 was one of the top 20 scoring siRNAs from the screen and contains the hit seed sequence ACTTGA shared between three other siRNAs from the top 20 scoring siRNAs as well as the siSharpin.1 siRNA used to confirm Sharpin as a hit in the kinase and phosphatase screen. This seed is also found in the miRNA miR-26a. siINADL.1 also shares its heptamer seed with one other top 20 scoring siRNA. This evidence suggests that at least part of the effect of siINADL.1 on TRAIL-induced cytotoxicity/Caspase-3/7 activity could be due to off-target effects. This is supported by the fact that siINADL.1 has a minimal effect on INADL transcript levels, although the fact that siINADL.2 increases the level of INADL transcript suggests that there is possibly some problem with the qPCR data here (Figure 5.9b). Set against this, siINADL.3, an independent siRNA that was not selected from the screen and does not contain a hit or enriched seed, did significantly reduce both the level of INADL transcript and TRAIL-induced Caspase-3/7 activity (Figure 5.9). Also the 3' UTR of INADL contains matches to all 5 hit seeds with the third highest frequency of matches to any of these seeds. This suggests that this gene at least warrants further investigation, even if the authenticity of its involvement in the regulation of TRAIL-induced apoptosis is currently unclear.

INADL is the human homolog of the *Drosophila* gene Inactivation No Afterpotential D (*Ina-D*). It contains 9 distinct PDZ domains (Pfam, Finn et al. 2006), which are domains

involved in protein/protein interaction. INADL is found at tight junctions in polarised cells (Lemmers et al. 2002) and has shown to be involved in directional migration of epithelial cells (Shin, Wang & Margolis 2007). *Ina-D* the *Drosophila* homolog of INADL is a scaffold protein involved in the organisation of signalling complexes at the cell membrane (Tsunoda, Zuker 1999). Phosphorylation of *frizzled* by atypical protein kinase C (*aPKC*) requires the *Ina-D* in drosophila cells, and it is hypothesised that *Ina-D* serves to anchor *aPKC* to *frizzled*. How INADL might affect the TRAIL pathway remains unclear.

5.8.3.5 TEGT

TEGT was isolated as a transcript homologous to a transcript expressed in the rat testis (Walter et al. 1994, Walter et al. 1995). The same gene was also isolated in a screen of human cDNAs in yeast which rescued Bax induced cell death (Xu, Reed 1998). TEGT was shown interact with Bax and protect against apoptosis induced by several triggers of the intrinsic apoptosis pathway, but not FAS (Xu, Reed 1998). TEGT has been shown to up-regulated in breast cancer cells, and that its knock-down by RNAi leads to spontaneous apoptosis (Grzmil et al. 2006). Surprisingly expression of the *Arabidopsis* homolog of TEGT in human cells triggers a cell death which is blocked by overexpression of XIAP, an effect which could be due to a dominant negative effect on endogenous TEGT (Yu et al. 2002). TEGT is isolated here as a gene whose knock down protects cells from TRAIL-induced apoptosis. However, several other genes have been isolated that have both pro and anti-apoptotic functions.

TEGT was targeted by two siRNAs which cause a significant reduction in TRAIL-induced Caspase-3 activity. Both of these siRNAs are from the library and both contain seeds from the hit seed set. Taking into account the known biological functions of TEGT, caution must be taken in concluding that TEGT is a positive regulator of TRAIL-induced apoptosis until further confirmation experiments have been carried out.

5.8.4 Conclusions

An siRNA screen was executed to identify genes from the druggable genome which are involved in the regulation of TRAIL-induced apoptosis. The screen accurately identified a number of siRNAs which reproducibly affected the sensitivity of cells to TRAIL. Confirmation experiments allowed for the genes targeted by some of these siRNAs to be identified as novel regulators of TRAIL –induced apoptosis. These genes are from diverse cell pathways.

A screen of this size is a major undertaking, requiring a large amount of resources. The majority of the resources consumed by the screen are in the form of assay reagents and consumables such as plates, pipette tips and media rather than in the form of the siRNA library itself. It is also a major undertaking in terms of time. However, the cost, in both time and materials, of the confirmation and follow up work is as much, if not more than that of the actual screen. Several decisions were taken to save time and materials such as performing only two replicates of the screen. It is worth considering the question of if these decisions were necessary, the cost of performing the screen can be justified. While expensive, time consuming and possibly imperfect, the screen, together with the accompanying follow-up work did identify several genes that are unlikely to have been identified by other means.

Worryingly several genes were targeted by two siRNAs that significantly reduced the level of TRAIL-induced Caspase-3/7 activity but were classified as off-targets due to the lack of phenotypic activity of a third siRNA which reduced the level of targeted transcript more efficiently. This suggests that screening hits should be confirmed by more than two siRNAs, and that results from RNAi experiments should ultimately be confirmed by non-RNAi experiments.

That results should be confirmed by more than two siRNAs, or at least an additional two siRNAs from those used in the screen is also suggested by examination of the seed sequences of high scoring siRNAs. Several seed sequences appear more than once in siRNAs targeting the genes targeted by the top 20 siRNAs, or in siRNAs used to confirm hits from the kinase and phosphatase screen, and additional seed sequences were found that were enriched in high scoring siRNAs. This suggests that process of screening is enriching for siRNAs with relevant off-target effects as well as relevant on-target effects. Analysis of the average frequency of these seeds in 3' UTRs suggests that siRNAs that contain "enriched" seeds may induce more off-target effects than other seed sequences. In contrast "hit" seed sequences were not found at a higher average frequency in 3' UTRs generally or specifically the 3' UTRs of genes associated with the TRAIL pathway with the exception of the seed ACTTGA, many copies of which is found in the 3' UTRs of several genes associated with the TRAIL pathway and is also the seed sequence of the human miRNA miR-26a.

A large number of the seed sequences found in the library match the 3' UTR of genes associated with TRAIL-induced apoptosis. This holds even when a smaller, higher quality list of TRAIL associated genes is used. This suggests that many of the siRNAs in the library have the potential to affect the level of genes previously associated with the TRAIL pathway. In this case the effect of an siRNA on TRAIL-induced cytotoxicity would be the

sum of all of its off-target effects and its on-target effects. The highest scoring siRNAs would be those that affected TRAIL-induced cytotoxicity through both on- and off-target effects. This is supported by the confirmation of some genes targeted by siRNAs containing these seeds, suggesting that at least some of the effect of the original siRNA was due to on-target effects.

6 AN OVEREXPRESSION SCREEN OF ORFs ON CHROMOSOME 22

In the preceding chapters the assay developed at the start of this thesis was applied in two RNAi screens. The benefits and drawbacks to this approach were discussed, particularly with relevance to the reliability and specificity of the results obtained. In an RNAi screen, the involvement of a gene in a process is assessed by studying the effect of reducing the level of its transcript. This is equivalent to studying loss-of-function mutations (with null/hypomorphic alleles of the gene in question) in traditional genetics. The role of a gene

in a process can also be implied by studying the effect of increasing the levels of a transcript. In traditional genetics, this is equivalent to studying gain-of-function mutations (with hypermorphic alleles of the gene in question). Prior to the use of RNAi this was the only form of genetic screening available in the non-sexual, diploid, mammalian cell culture system. Random mutagenesis can be used to screen for gain-of-function mutants as they tend to be dominant. A more common approach is to introduce libraries of cDNA clones generated by the reverse transcription of RNA, isolated from tissue or cell lines into cell lines and selecting for clones that induce the desired phenotype. Once the clone is identified it can be sequenced to identify its source. Indeed one group has taken this approach to identifying regulators of the TRAIL-induced apoptosis pathway, identifying two known apoptosis inhibitors from a library of HepG2 cDNAs (Burns, El-Deiry 2001). However, such strategies usually involve, to a greater or lesser extent, some amount of pooling of clones and therefore will suffer from the problems with such a strategy outlined earlier in this thesis. Most importantly, the clone in question must induce a very large effect compared to the background level in order to be identified in a selective screen. Furthermore, such cDNA libraries are generally of an unknown complexity, with the make up being dependent on the complement of transcripts expressed in the source material. They will also contain truncated and mis-spliced transcripts. The availability of high-quality annotations of genomes allows for the construction of libraries of clones that contain one, sequence verified, clone for each full-length open reading frame (ORF) in a genome (the ORFeome, (Brasch, Hartley & Vidal 2004)). Such collections may be used to conduct reverse genetic screens for the effect of over-expression of these ORFs, in a one-well-one gene manner.

Here a pilot of such an approach is described. Plasmids driving the over expression of each of 288 full length ORFs from chromosome 22 are introduced individually into HeLa cells, and their effect on TRAIL-induced apoptosis assessed.

6.1 The Chromosome 22 ORF collection

The Chromosome 22 ORF collection used in this chapter is a collection of clones corresponding to 288 of the 398 (72%) predicted full-length open reading frames identified on chromosome 22 (Collins et al. 2003, Collins et al. 2004). The ORFs were cloned using an annotation driven approach. Many large scale ORF cloning efforts are based on sequencing full-length cDNA collections (which include 3' and 5' UTRs), selecting a clone to represent each gene and sub-cloning the ORF. An annotation driven approach involves creating PCR primers directed to each annotated ORF, amplifying the ORFs from cDNA pools and

cloning them into a sequencing vector. Clones containing the ORF are then sequenced and compared to the sequence predicted from the annotations. Clones with base changes that are not previously reported Single Nucleotide Polymorphisms (SNP), and clones that are splice variants which do not maintain the reading frame of the ORF are rejected. This leads to a single, sequence verified clone for each ORF (Figure 6.1). ORFs in the chromosome 22 ORF collection were cloned both with and without stop codons and then sub-cloned into holding and expression vectors (with either a C or N terminal T7 tag) compatible with Invitrogen's Gateway cloning technology.

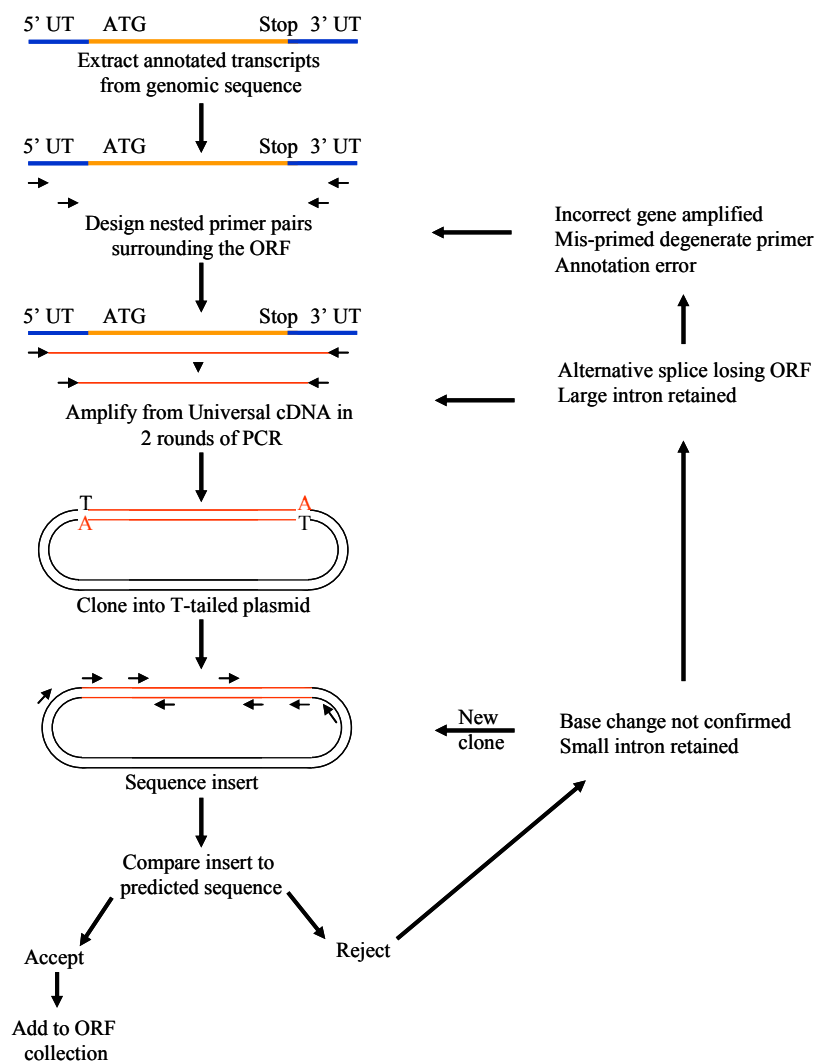


Figure 6.1 An annotation driven ORF cloning strategy (from Collins et al. 2004)

ORFs are tagged at both C and N terminals in separate constructs to control for effects of the tag on the function or localisation of the resulting protein. It was reported that tagging ORFs at the C terminal is generally more reliable than tagging at the N terminal, presumably because tagging at the N terminal would mask any localisation sequences at this terminus (Palmer, Freeman 2004, Simpson et al. 2000). However, others have found that

tagging at the N terminal is at least as good as or better than tagging at the C terminal (J. Collins, personal communication).

6.1.1 Gateway cloning

The Gateway cloning system utilises modified enzymes from bacteriophage λ to clone fragments and allow the movement of fragments between vectors using homologous recombination, without restriction digestion, purification and ligation. Inserts are generated by PCR amplification using primers tagged with a bacteriophage λ recombination site (attB). A modified enzyme from bacteriophage λ (BP clonase) is then used to recombine the attB sites with the bacteriophage λ recombination site attP in a donor vector to form an “entry” clone. The recombination leads to the attB and attP sites forming composite attL and attR sites, with the attL sites flanking the insert. This recombination removes a ccdB gene from the donor vector and allows selection against unrecombined vectors in bacterial strains in which the ccdB gene is toxic. Inserts can be sub-cloned into another vector which contains two attR sites flanking a ccdB gene, known as a destination vector, in a second recombination reaction using the LR clonase enzyme.

The chromosome 22 ORF collections is available cloned into the pGEM holding vector, as entry clones cloned into the donor vector pDONR223, and cloned into an expression vector based on pCDNA3, with a T7 epitope fused at either to C or N terminal and expression of the ORF driven from the CMV promoter. The expression vectors are arrayed in six 96-well plates, three with C terminal tags (plates 1,3 and 5) and three with N terminal tags (plates 2,4 and 6).

6.2 Design and execution of screen

DNA was successfully prepared from 555 of the 576 clones which comprise the 288 chromosome 22 ORF set cloned in expression vectors, tagged at both the C and N terminals. While several genes exist for which it is known that overexpression leads to a reduction in TRAIL sensitivity (e.g. the gene for the anti-apoptotic protein cFLIP, Inhibitor proteins such as XIAP, cIAP1 or cIAP, or indeed the TRAIL decoy receptors), no such clones are present in this ORF set, and are not easily obtainable in the correct format. Due to a lack of the time required to clone and tests such a construct, the screen was conducted without a positive control. Choice of such a negative control is difficult. The standard control in such experiments would be an empty vector. However, it is unclear if this is a suitable negative control, as it does not control for the effects of producing large amounts of

protein. Another option is to select a protein which is not expected to be involved in the process. One option would be a non-native protein such as GFP or Luciferase. This would not control for the effects of large amounts of endogenous protein being produced however. Another option is to choose a native protein which is not predicted to be involved. In order to select such a protein as truly not having an effect on TRAIL-induced cytotoxicity a panel of such proteins could be tested. Since the majority of ORFs are not expected to affect the sensitivity of cells to TRAIL-induced cytotoxicity, measuring against the baseline effect seen in the majority of clones is effectively equivalent to testing a panel of potential negative controls which includes all the constructs in the library. .

DNA prepared from the 555 chromosome 22 ORFs containing clones was transfected in duplicate into HeLa cells (passaged four times since defrosting) and assayed for sensitivity to 0.5 μ g/ml TRAIL using alamarBlue. Plates were processed in batches of three plates per experiment.

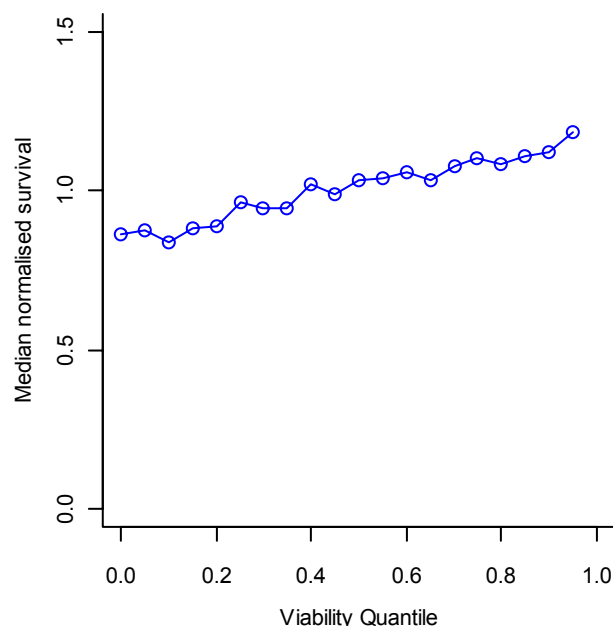


Figure 6.2 Relationship between pre-treatment viability and sensitivity to TRAIL-induced cytotoxicity
Viability of cells in each well prior to treatment was normalized to plate median viabilities. Normalised viabilities were divided into 20 quantiles. The median normalized post treatment survival was calculated for wells in each of these quantiles.

It has been previously observed that the density of cells at time of treatment affects the sensitivity of cells to TRAIL-induced apoptosis. In the previous chapter, the relationship between pre-treatment viability and post-treatment survival was examined to establish a pre-treatment viability cut off, below which results would be removed from further analysis (Figure 5.2). This examination showed a sharp drop in post-treatment survival in wells which scored in the bottom 20% for pre-treatment viability. For the results from the ORF screen no such sharp drop was observed (Figure 6.2). There is an increase in normalized

survival with increasing pre-treatment viability across the whole range of pre-treatment viability, although the range of median normalized survival is much smaller than is the case for kinase and phosphatase RNAi screen. The relationship observed can not be removed by applying a cut-off to the data. In order to remove those data points where the pre-treatment survival is so low as to cause problems for data analysis the wells with the 5% lowest pre-treatment viability were removed from further analysis.

Data was normalised using the median survival for each plate. Figure 6.3 shows the effects of this normalization. The difference between different batches of plates processed on different days, using different batches of cells, can be clearly seen in the unnormalised data (Figure 6.3a), while no such differences are apparent in the normalised data (Figure 6.3b).

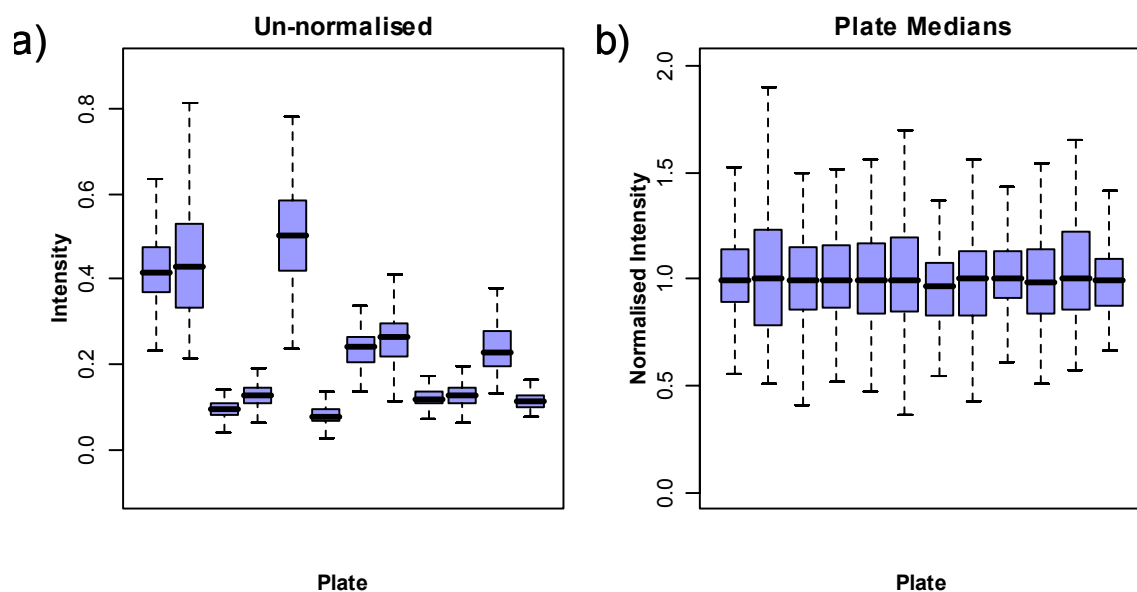


Figure 6.3 Normalisation of data from over-expression screen of Chromosome 22 ORFs

a) Boxplot of raw survival data from screen on a per plate basis. b) Boxplot of survival data normalized to plate median survival on a per plate basis

The distribution of data from previous screens was shown to be non-normal. A link was demonstrated between mean survival and standard deviation between replicates. Log transformation of the data did not abolish this relationship, instead inverting it. In the case of the data from the siRNA screen of the druggable genome, while log transformed data showed a relationship between mean survival and standard deviation between replicates, the relationship was weaker than for non-transformed data. The mean survival rank of data from the over-expression screen was plotted against the standard deviation between replicates for both untransformed and log transformed data (Figure 6.4). A relationship was observed between mean survival rank and standard deviation in untransformed data (Figure 6.4a). This relationship is less pronounced in log transformed data (Figure 6.4b).

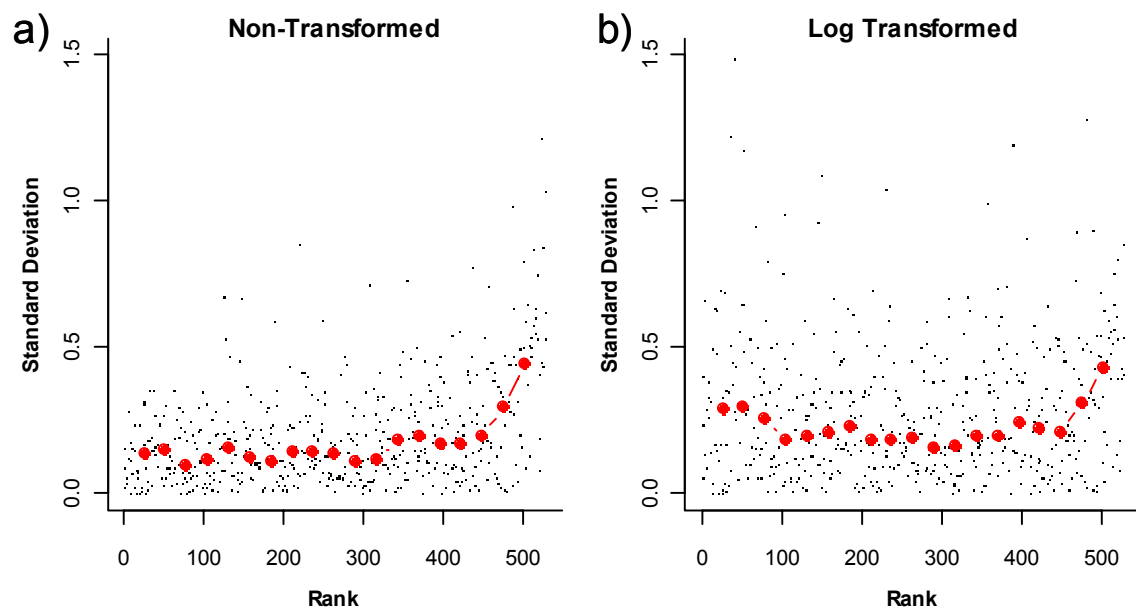


Figure 6.4 Relationship between rank of mean and standard deviation between replicates of Chromosome 22 ORF expression screen

For each expression clone the mean of the normalized data for each replicate was calculated. The rank of this mean was then plotted against the standard deviation between the replicates for a) Non-transformed data and b) Log transformed data. The red line in each plot represents the running median standard deviation.

Data were analysed using the R/Bioconductor package cellHTS, first excluding wells with a low pre-treatment viability, and then median normalizing plates with a log transformation, and using the minimum of replicates as a summary function.

6.3 Screen Results

Without controls, an analysis of the quality of the screen is restricted. There is very little correlation between the two replicates of the screen (Figure 6.5a). The correlation coefficient is 0.32 ($r^2 = 0.10$). This means that variation caused by random variation between replicates is greater than the variation caused by the effect of the expression of the ORF on the survival of TRAIL-treated cells. This could be due to a large amount of random variation. Alternatively it could suggest that the majority of the ORFs have little or no effect of the survival of cell treated with TRAIL. The correlation between the N and C terminal tagged version of the same ORF is slightly lower (Figure 6.5b), with a correlation coefficient of 0.22 ($r^2 = 0.048$). This could suggest that there is a real difference between ORFs tagged at different ends, or alternatively that again, the effects of random variation are higher than effects caused by the expression of the ORFs. One reason for the lack of effect could be that the ORFs are not expressed from the transfected constructs. This is unlikely however as transfection of the constructs from the same preparations into COS cells, immunofluorescent staining showed expression of 73% of genes (J. Collins, manuscript in

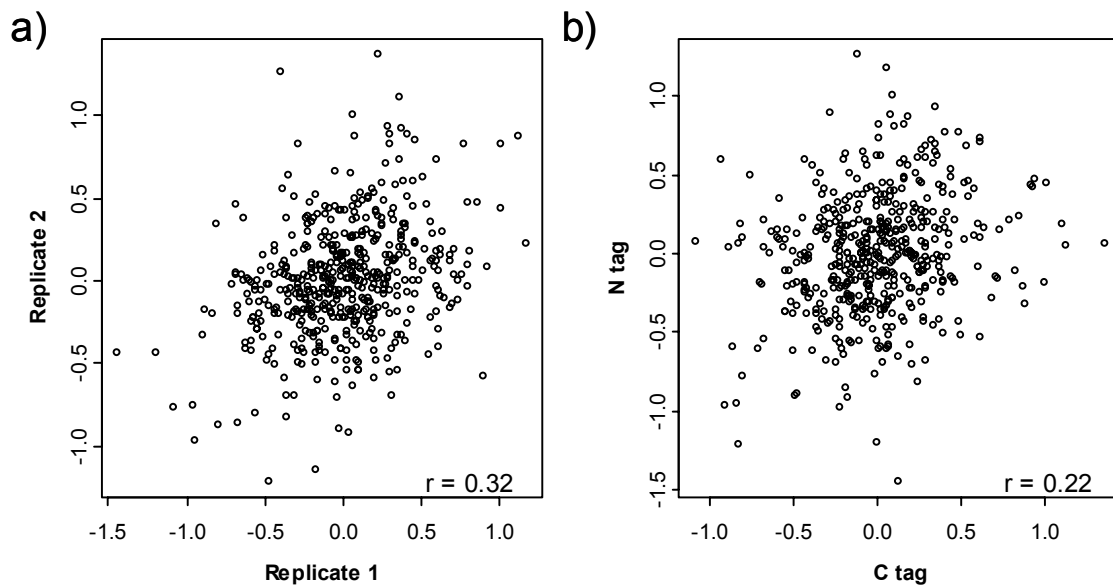


Figure 6.5 Correlations in data from chromosome 22 ORF expression screen

a) Normalized survival from replicate 1 plotted against normalised survival from replicate 2. b) Plot showing normalised survival of the two constructs expressing the same ORF, tagged at either the C or N terminal. The Pearson's correlation co-efficient is shown in the bottom right corner of each plot. preparation).

The distribution of scores from the screen is roughly normal (Figure 6.6a and Figure 6.6b). Examination of the distribution of well scores across and between plates reveals no obvious position dependent effects (Figure 6.6c).

ORFs were ranked according to their score in the screen. A portion of this ranking is shown in Table 6-1. Examination of the quartile-quartile plot of the data, which plots the actually quartile of a datum point against the theoretical quartile were the data from a normal distribution, shows four points clearly score higher than would be expected if the data were normally distributed (blue points, Figure 6.6b). These points correspond to the four highest scoring clones, those expressing C-terminal tagged RBX1 and AIFM3 and the N-terminal tagged LIMK2 and MTMR3 (Table 6-1).

6.4 Confirmation of Hit genes

The four ORFs which scored significantly higher than other ORFs in the screen (RBX1, AIFM3, LIMK2 and MTMR3) were selected for confirmation. In each case only one of the two clones containing each ORF scored highly in the screen, although the clone containing the second RBX1 clone also appeared in the top ten clones (Table 6-1). This could be due to interference from the T7 epitope tag when at one end of the ORF, but not the other. To avoid interference from the tag, the ORFs were transferred into an expression vector containing no tag.

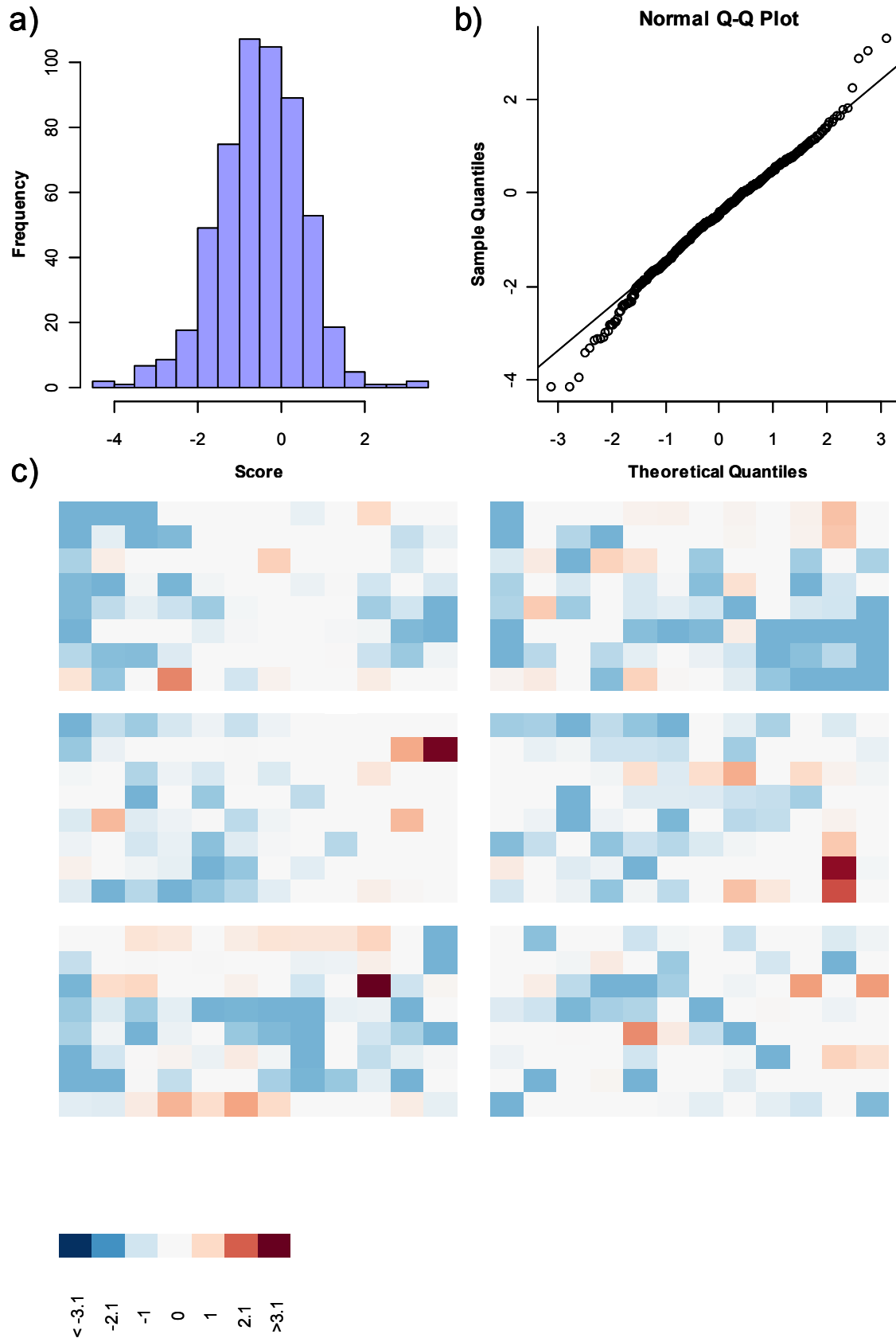


Figure 6.6 Scores of clones from screen of chromosome 22 ORFs

a) Histogram showing distribution of scores. b) A normal Quartile-Quartile plot of scores. The actual quantile of a data point is plotted against the theoretical quartile of that point if the data were normally distributed. If the data were perfectly normally distributed all data would fall on the line shown. c) A heat map showing the scores of each well from the screen. High scoring wells are shown in red, low scoring wells in blue.

Gene ID	Gene Symbol	Tag	Description	Normalised Survival		score
				Rep 1	Rep 2	
9978	RBX1	C	Ring-box 1	NA	0.948	3.26
150209	AIFM3	C	Apoptosis-inducing factor, mitochondrion-associated, 3	1.129	0.876	3.01
3985	LIMK2	N	LIM domain kinase 2	1.007	0.824	2.83
8897	MTMR3	N	Myotubularin related protein 3	0.771	0.825	2.22
129138	ANKRD54	C	Ankyrin repeat domain 54	0.625	NA	1.8
10478	SLC25A17	N	Solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein, 34kDa), member 17	0.606	0.738	1.74
10740	RFPL1S	N	Ret finger protein-like 1 antisense	0.856	0.474	1.63
9978	RBX1	N	Ring-box 1	0.794	0.468	1.61
23765	IL17RA	C	Interleukin 17 receptor A	0.541	0.46	1.56
150280	HORMAD2	C	HORMA domain containing 2	1.012	0.436	1.5
9514	GAL3ST1	N	Galactose-3-O-sulfotransferase 1	0.513	0.626	1.48
758	MPPED1	C	Metallophosphoesterase domain containing 1	0.491	NA	1.41
2130	EWSR1	C	Ewing sarcoma breakpoint region 1	0.469	0.851	1.35
66035	SLC2A11	C	Solute carrier family 2 (facilitated glucose transporter), member 11	NA	0.392	1.35
468	ATF4	N	Activating transcription factor 4 (tax-responsive enhancer element B67)	0.448	0.449	1.29
57591	MKL1	N	Megakaryoblastic leukemia (translocation) 1	0.444	0.598	1.28
84133	ZNRF3	N	Zinc and ring finger 3	0.559	0.355	1.22
51512	GTSE1	N	G-2 and S-phase expressed 1	0.415	0.884	1.2
1399	CRKL	N	V-crk sarcoma virus CT10 oncogene homolog (avian)-like	0.415	0.506	1.19
150290	DUSP18	C	Dual specificity phosphatase 18	0.395	0.346	1.14

Table 6-1 Top scoring clones from chromosome 22 ORF over-expression screen

Table shows the 20 highest scoring clones from the over-expression screen of ORFs from the chromosome 22 ORF collection. Gene ID is the EntrezGene ID for the gene and Tag indicates the terminal at which the ORF is tagged with a T7 epitope. Complete ranking can be found on included CD or online at <http://www.sanger.ac.uk/HGP/Chr22/ORFScreen.txt>

No Gateway compatible expression vectors are available that do not contain a tag fused to either terminal of the ORF. To allow analysis of the effect of expression of hit ORFs free from tag dependent effects a Gateway compatible expression vector containing no tag was created. First the gateway cassette was removed from the pCDNA3.GW.V5N vector by digestion, relegation and transformation into a ccdB sensitive strain to select against gateway cassette containing plasmids. The V5 tag was then removed by digestion. The gateway cassette was then reintroduced and the vector transformed into a ccdB insensitive strain. Multiple colonies resulting from the transformation were cultured and plasmid DNA prepared. The orientation of the gateway cassette within these clones was verified by restriction digestion. This new vector was named the pCDNA3.GW.NoTag vector.

Gateway recombination was used to transfer ORF inserts identified in the screen from the entry clones into the pCDNA3.GW.NoTag vector. The identities of the ORFs were confirmed by DNA sequencing.

To confirm the effect of over-expression of the 'hit' ORFs on sensitivity of cells to TRAIL-induced cytotoxicity, cells were transfected with pCDNA3.GW.NoTag constructs containing each of the ORFs and tested for sensitivity to a range of TRAIL concentrations (Figure 6.7). While in the screen, the majority of constructs which had no effect on TRAIL sensitivity could be used as a negative control, this is not possible here, since all the clones could be expected to score highly. Therefore, despite the issues raised earlier, the empty vector pCDNA3.T7 was used as a negative control. In addition the ORF of the gene PICK1 was also transferred into the NoTag vector. PICK1 had a low score (-2.34) in the screen and serves as a second negative control. The pSM2.shCasp8.2 construct, which expresses a hairpin targeting Caspase-8, was used as a positive control.

Transfection of the positive control, pSM2.shCasp8.2 caused a modest increase in survival compared to the empty pCDNA3.T7 vector at all concentrations of TRAIL tested, with 19% of pSM2.shCasp8.2 transfected cells surviving treatment with 1µg/ml TRAIL compared with 11% of vector transfected cells. This compares with an increase in survival from 29% to 40% seen previously (Figure 3.11). Transfection of clones expressing PICK1 has survivals very similar to the empty vector (12% of PICK1 transfected surviving treatment with 1µg/ml TRAIL compared with 11% of vector transfected cells, Figure 6.1e). Transfection of clones expressing RBX1 leads to a decrease in sensitivity to TRAIL at all concentrations tested (Figure 6.7a), with 30% of cells surviving treatment with 1µg/ml

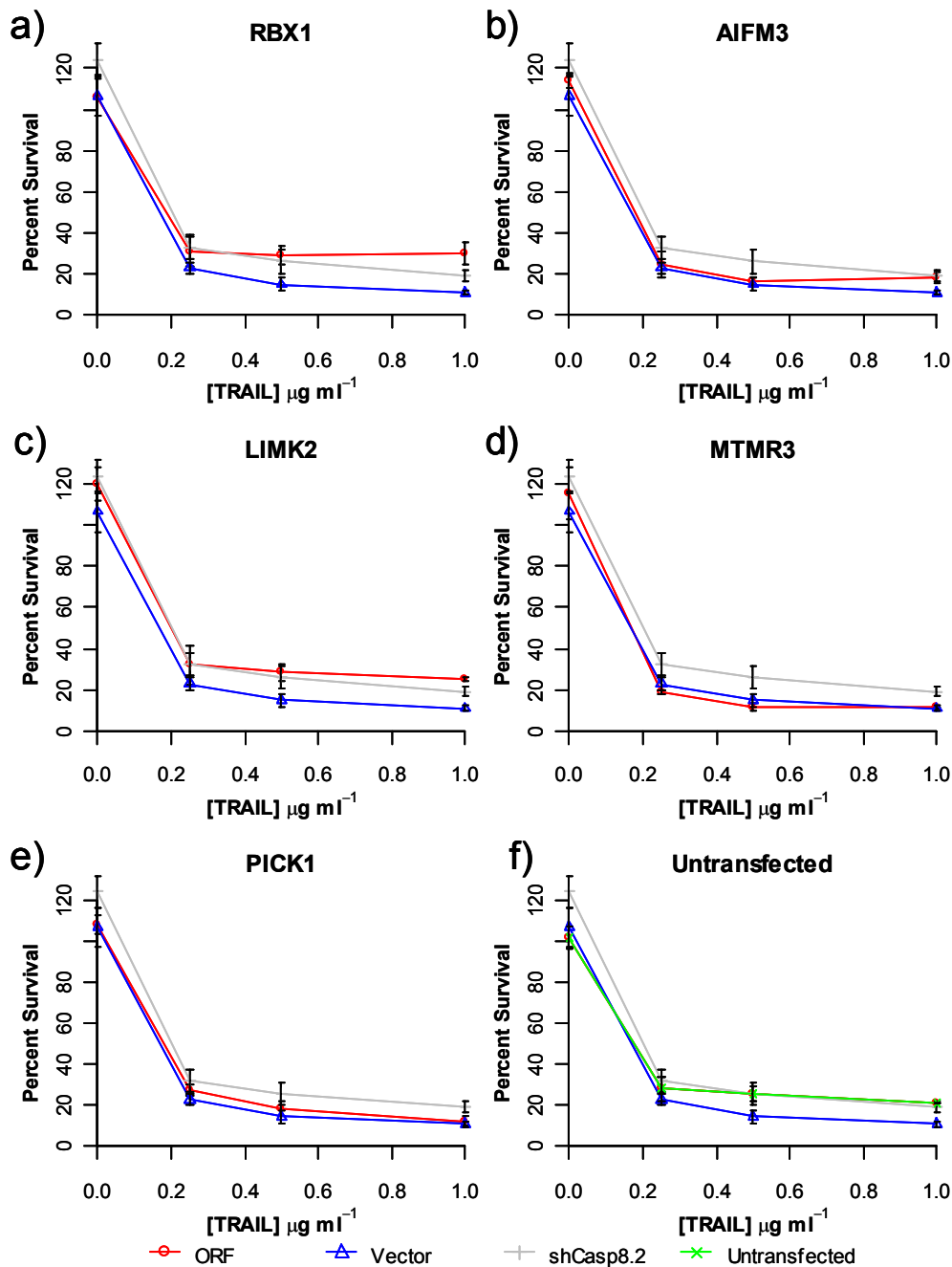


Figure 6.7 Effect of transfection of candidate hit ORFs on sensitivity to TRAIL-induced cytotoxicity
Cells were transfected with either expression clones for a) RBX1, b) AIFM3, c) LIMK2, d) MTMR3, e) PICK1, pSM2.shCasp8.2 as a positive control, or empty pCDNA3.T7 vector as a negative control, or f) mock transfected. Viability was assessed 48 hours later and cells were treated with the concentration of TRAIL indicated for 24 hours and viability reassessed. Error bars represent 1 standard deviation, n=3

TRAIL compared to 11% of cells transfected with vector. Similarly transfection of cells with the construct expressing LIMK2 caused a reduction in sensitivity at all concentration compared to transfection with an empty vector, with 25% and 11% of cells transfected with LIMK2 or empty vector respectively surviving treatment with $1\mu\text{g/ml}$ TRAIL (Figure 6.7c). A difference between cells transfected with the construct expressing AIFM3 and empty vector was not seen at lower concentrations of TRAIL, however more AIFM3 expressing

cells survived treatment with 1 μ g/ml than cells transfected with empty vector (18% compared with 11%, Figure 6.7b). In all cases these differences at 1 μ g/ml TRAIL are significant at the 5% significance level (p values calculated by Bonferroni corrected Student's t -test on log transformed data). No difference was seen between pCDNA3.MTMR3 transfected cells and vector transfected cells at any concentration of TRAIL (Figure 6.7d). As was seen in previous chapters, mock transfected cells were less sensitivity than negative control transfected cells at all concentrations of TRAIL tested (Figure 6.7f).

Worryingly transfection of several of the clones increases the survival of untreated cell as well as TRAIL treated cells when compared to control transfected cells (although in no individual case is this difference significant at the 5% level). This raises the possibility that the effect of the clones on the survival of cells compared to the negative control could be due to a growth advantage, or some other, non-TRAIL specific effect. In order to address this possibility, the data were renormalized by dividing all survival values by the survival of untreated cells, thus expressing survivals as a proportion the untreated cells which survived treatment (Figure 6.8). With this new normalisation, the difference between RBX1 and LIMK2 transfected cells and negative control transfected remain statistically significant at the 5% level. However, the difference between normalised survival for AIFL transfected cells and normalised survival for negative control transfected cells is not significant at the 5% level (survival of AIFL transfected cells treated with 1 μ g/ml TRAIL was 15% of that in untreated cells, compared with 10% for negative control transfected cells).

These data show that transfection with constructs expressing three of the four ORFs identified from the screen do reduce the sensitivity of cells to TRAIL-induced cytotoxicity. The finding that untransfected cells are also less sensitive to TRAIL-induced cytotoxicity raises the possibility that the effect seen for these ORFs might not be due to the over expression of the particular ORF. Specifically it is possible that some property of these constructs is preventing their entry into cells, preventing the expression of the ORF from the construct or killing transfected cells, such that at the time of treatment, the cells are not expressing the ORF being tested. To rule out the possibility that transfection of these constructs was killing the transfected cells, the pre-treatment viabilities of cells transfected with each construct were compared. There was no difference between the viability of cells transfected with any of the pCDNA3.ORF constructs compared to cells transfected with the empty vector pCDNA3.T7, suggesting that the difference was not due differences in toxicities between transfections with these different constructs (data not shown). A small, but highly statistically significant difference was seen between the viabilities of mock-

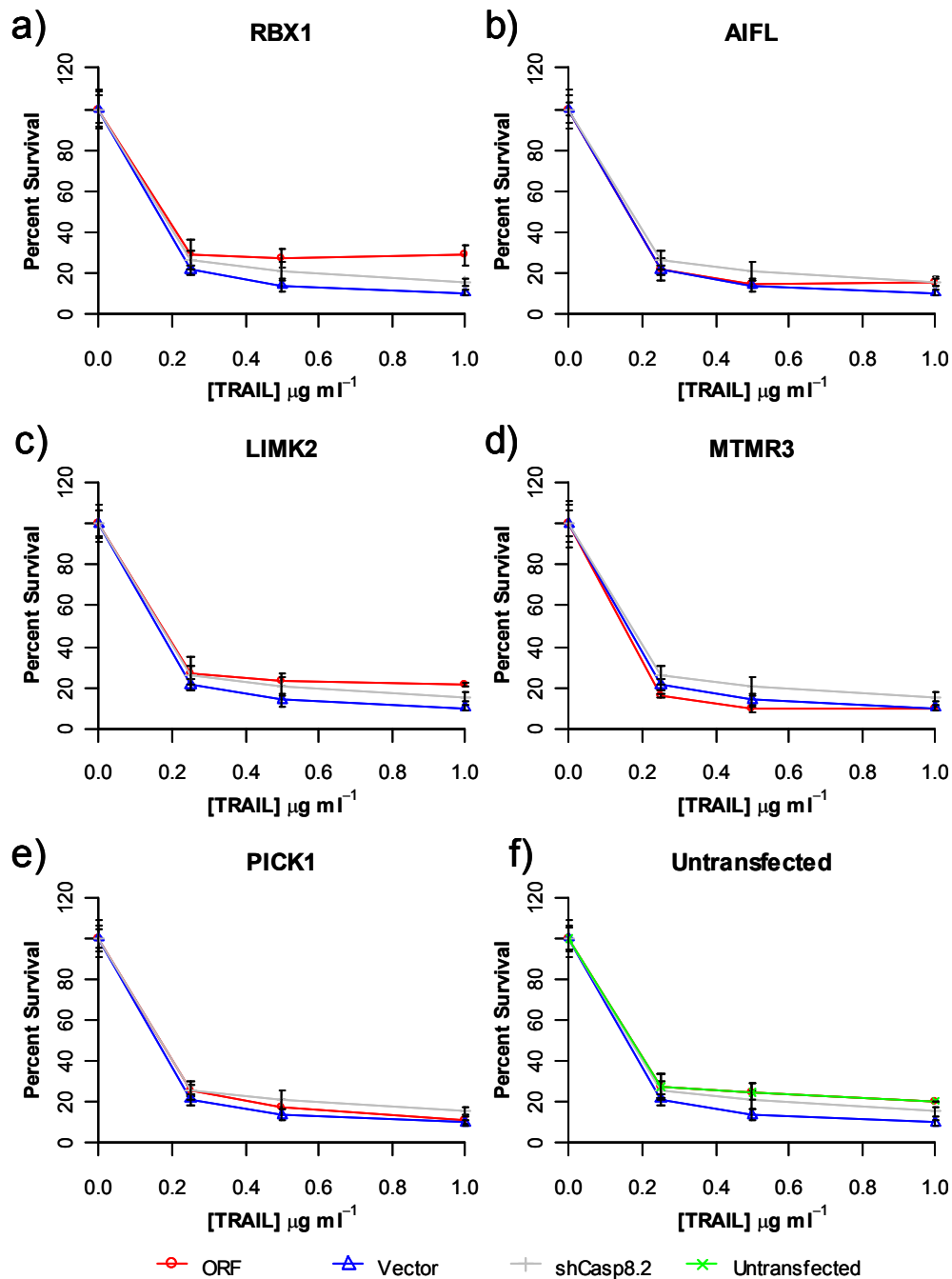


Figure 6.8 Renormalised effect of transfection of candidate hit ORFs on sensitivity to TRAIL induced cytotoxicity

Experiment was carried out as described for Figure 6.7. Data is presented as survival relative to untreated cells. transfected and negative controls transfected cells (viability of mock transfected cells was 110% that of cells transfected with the negative control, $p = 0.001$ by Students' t -test, $n = 12$). Thus difference in the TRAIL sensitivity of cells transfected with hit ORF expressing constructs and negative control transfected cells is not due to the constructs killing transfected cells. It is possible that the increased survival of mock-transfected cells compared to non-transfected cells could be due to the toxicity of transfection, as it was shown that sensitivity to TRAIL is linked to pre-treatment cell number (Figure 6.2).

These data show that transfection with constructs expressing three of the four ORFs identified from the screen do reduce the sensitivity of cells to TRAIL-induced cytotoxicity. The finding that untransfected cells are also less sensitive to TRAIL-induced cytotoxicity raises the possibility that the effect seen for these ORFs might not be due to the overexpression of the particular ORF. Specifically it is possible that some property of these constructs is preventing their entry into cells, preventing the expression of the ORF from the construct or killing transfected cells, such that at the time of treatment, the cells are not expressing the ORF being tested. To rule out the possibility that transfection of these constructs was killing the transfected cells, the pre-treatment viabilities of cells transfected with each construct were compared. There was no difference between the viability of cells transfected with any of the pCDNA3.ORF constructs compared to cells transfected with the empty vector pCDNA3.T7, suggesting that the difference was not due differences in toxicities between transfections with these different constructs (data not shown). A small, but highly statistically significant difference was seen between the viabilities of mock-transfected and negative controls transfected cells (viability of mock transfected cells was 110% that of cells transfected with the negative control, $p = 0.001$ by Students' t-test, $n = 12$). Thus difference in the TRAIL sensitivity of cells transfected with hit ORF expressing constructs and negative control transfected cells is not due to the constructs killing transfected cells. It is possible that the increased survival of mock-transfected cells compared to non-transfected cells could be due to the toxicity of transfection, as it was shown that sensitivity to TRAIL is linked to pre-treatment cell number (Figure 6.2).

To ensure that transfection with the ORF expressing clones is driving expression of these ORFs at higher than normal levels, the expression of the ORFs in cells transfected with both fresh preparations of the T7 tagged constructs used in the screen and the NoTag constructs was measured using qRT-PCR (Figure 6.9). Unfortunately, despite multiple attempts, DNA could not be prepared for the N-terminal tagged MTMR3 ORF. Since the cloned ORFs do not contain introns, it is not possible to design oligonucleotide primers that will amplify from mRNA, but not DNA. Therefore, to control for the presence of plasmid DNA from the transfection, RNA was prepared in a mock reverse-transfection reaction. qPCR on this RT⁻ sample would amplify from DNA only, therefore allowing an estimate of the proportion of the increase in RNA expression measured in the reverse-transcribed samples (RT⁺) that is due to contamination of the RNA extraction with DNA. The majority of the samples were shown to be contaminated with DNA. However, DNA contamination made up only a small part of the increase in product amplified when transfected with ORF

expression construct. For example, there was an 87 fold increase in amplification of AIFM3 in the RT⁻ sample prepared from pCDNA.AIFM3.T7C transfected cells compared to cDNA from the negative control sample. However there is a 1,257 fold increase in amplification of AIFM3 in the RT⁺ sample prepared from the same transfection compared to the negative control sample.

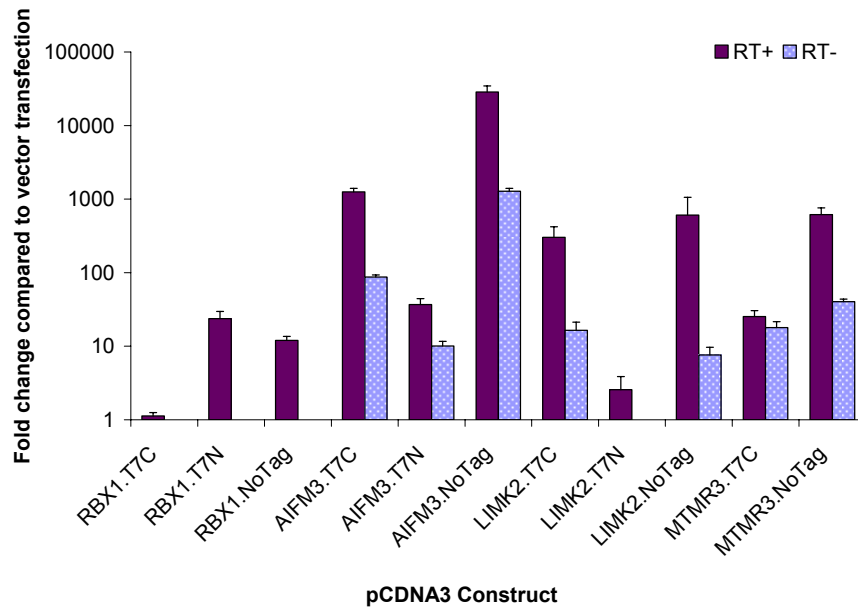


Figure 6.9 Expression of hit ORFs in cells transfected with ORF expressing constructs

RNA was isolated from cells transfected with constructs which express the 'hit' ORFs, either tagged with T7 epitope at the C or N terminal or not tagged, or pCDNA3.T7 as a negative control. RNA was either reverse transcribed to generate cDNA (RT⁺), or mock reverse transcribed in a reaction lacking reverse transcriptase (RT⁻). qPCR was performed on the samples using oligonucleotide primers designed to amplify either from the ORF or from GAPDH or ACTB. Expression levels were calculated using a variation of the Pfaffl method to allow normalisation to multiple housekeeping genes (Hellemans et al. 2007). Levels of GAPDH and ACTB in RT⁺ samples were used to normalize all samples. Results are presented as fold increase compared to levels in the RT⁺ sample from the negative control transfected cells. Error bars represent 1 standard error of the mean. n = 3.

In all cases, with the exception of the RBX1.T7N and MTMR3.T7C expressing constructs, transfection with ORF expressing constructs led to an increase in the level of the transcript for that ORF. The largest increase was the increase in the levels of AIFM3 transcript in pCDNA3.AIFM3.NoTag transfected cells, which showed a greater than 28,000 fold increase in transcript levels compared to negative control transfected levels. Such a huge increase in transcript levels suggests that levels in negative control transfected cells are very low. By contrast, transfection with pCDNA3.LIMK2.T7N causes a 2.5 fold increase in transcript levels compared to negative control transfected cells.

These data show that transfection with constructs expressing three of the four candidate hit ORFs (RBX1, AIFM3 and LIMK2) does lead to a reduction in sensitivity to TRAIL, and that this reduction in sensitivity is accompanied by an increase in transcript

levels.

6.5 Characterisation of hit ORFs

6.5.1 Effect on TRAIL-induced Caspase activity

The effect of three of the ORFs identified in the screen of ORFs from chromosome 22 on TRAIL-induced cytotoxicity was confirmed. To investigate the involvement of these ORFs in TRAIL-induced apoptosis, and also map the position in the pathway at which they function, the effect of transfection of constructs expressing these ORFs on TRAIL-induced activation of Caspases was measured using luminescent caspase assays (Figure 6.10).

Treatment of negative control transfected cells with TRAIL induces a 2.5-fold increase in Caspase-8 activity (Figure 6.10a), a 3.0-fold increase in Caspase-9 activity (Figure 6.10b) and a 19% increase in Caspases-3/7 activity (Figure 6.10c). Transfection with pSM2.shCasp8.2 reduces the level of TRAIL-induced Caspase-8 and Caspase-9 activity to 78% of that in negative control transfected cells treated with TRAIL and Caspases-3/7 activity to 67% of that in negative control transfected cells. Note that this reduction in Caspase-3/7 reduces levels of caspase activity in pSM2.shCasp8.2 transfected cells to below that seen in untreated negative control transfected cells, but not below that seen in untreated pSM2.shCasp8.2 transfected cells.

Transfection of none of the pCDNA3.ORF constructs reduced the level of TRAIL-induced activity of Caspase-8, Caspase-9 or Caspases-3/7. The level of Caspase activity measured in non-TRAIL treated samples is similar for Caspases-8 and Caspase-9 irrespective of the construct with which the cells were transfected. The level of Caspase-3/7 activity measured in untreated samples does vary depending on the construct transfected. However, since knock-down of Caspase-8 does not reduce levels of Caspase-8 activity measured in untreated samples, it is possible that these levels are assay background rather than a measure of some level of caspase activity in the absence of an activator.

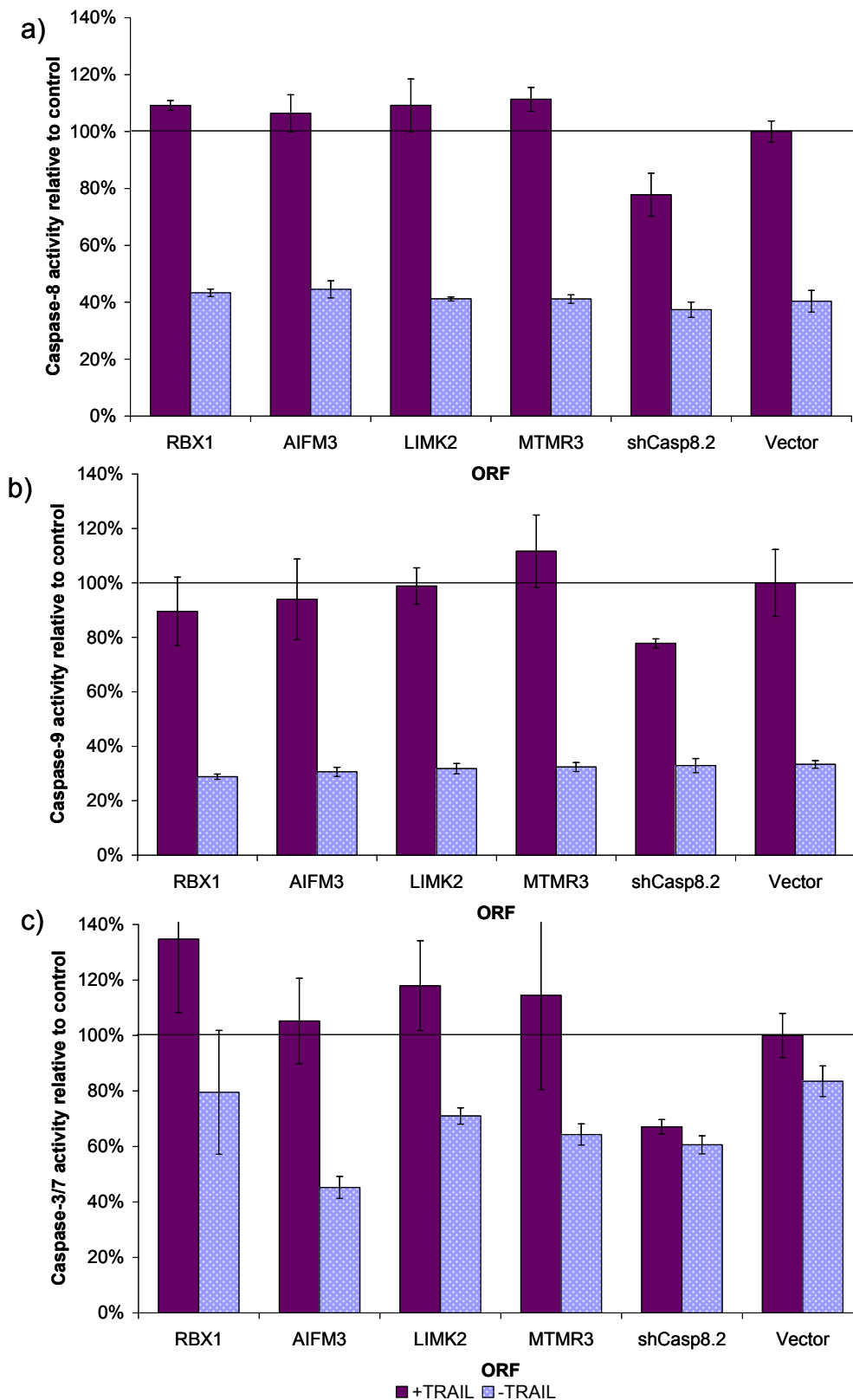


Figure 6.10 Effect of over-expression of hit ORFs on TRAIL-induced Caspase activity

Cells were transfected with pCDNA3.NoTag constructs expressing one of the hit ORFs or empty pCDNA3.T7 vector. 48 hours after transfection cells were treated with 0.5 μ g/ml TRAIL for 6 hours. a) Caspase-8, b) Caspase-9 or c) Caspase-3/7 activity was measured using Promega Caspase-Glo luminescent caspase assays. Results are expressed as percentage of caspase activity in TRAIL treated negative control transfected cells. In each case solid line represents 100% of control. Error bars represent 1 standard deviation, n = 3.

The assay used in the screen measures the effects of TRAIL-induced cytotoxicity rather than TRAIL-induced apoptosis. A key characteristic of apoptosis is the induction of Caspases. Evidence that expression of ORFs that reduce the level of TRAIL-induced cytotoxicity also reduce the levels of TRAIL-induced caspase activity would allow the conclusion that expression of such ORFs reduces TRAIL-induced apoptosis. No evidence was found for the ORFs identified from the screen and so no such conclusion can be drawn. One conclusion that could be drawn is that overexpression of these ORFs is affecting sensitivity of cells to TRAIL-induced cytotoxicity in a manner unconnected to apoptosis, although it must be noted that the data presented here do not necessarily demonstrate that.

6.5.2 Effect on sensitivity to other apoptosis inducing conditions

If expression of hit ORFs does in fact affect the sensitivity of cells to TRAIL-induced apoptosis, it could do at so several levels. They could affect the sensitivity of cells to ligand induced apoptosis (or purely TRAIL-induced apoptosis), or they could affect the sensitivity of cells to apoptosis in general. If this were the case it would be expected that expression of these ORFs would reduce the sensitivity of cells to non-ligand induced apoptosis as well as ligand induced apoptosis.

To test the sensitivity of cells to apoptosis induced by ligands other than TRAIL, the level of cytotoxicity induced by treatment with a range of concentrations of FAS was measured. Unfortunately minimal cell death was observed when negative control transfected cells were treated with any of the concentrations of FAS ligand tested (data not shown).

Exposure to H₂O₂ and UV radiation are two treatments with induce apoptosis through the intrinsic pathway, via oxidative stress and DNA damage respectively. In order to determine if the ORFs identified in the screen affect non-ligand induced apoptosis the effect of expression of hit ORFs on sensitivity to cytotoxicity triggered by these treatments was assessed (Figure 6.11 and Figure 6.12).

Treatment of cells with H₂O₂ caused cytotoxicity approximately proportional to the concentration of H₂O₂, with treatment with 100µM of H₂O₂ killing all of the cells and treatment with 50µM leading to a 36% survival rate. This was true for both negative control transfected and untransfected cells (Figure 6.11e). Expression of none of the hit ORFs changed the sensitivity of cells to H₂O₂ at any concentration tested (Figure 6.11a-d). The lack of a positive control in this experiment makes it difficult to assess the meaning of these results, as it is not possible to know if the experiment would show a clear effect for genes involved in the oxidative stress. An appropriate positive control would be a protein known to

be an inhibitor of the mitochondrial (intrinsic) apoptosis pathway, such as Bcl-2 or XIAP.

Exposure to UV radiation caused cytotoxicity to a large portion of cells, with 25% of negative control transfected cells surviving a 200 Jm⁻² dose (Figure 6.12). Unfortunately, as was seen in similar experiments testing the effect of transfection of hit siRNAs from the kinase and phosphatase screen on the sensitivity of cells to UV irradiation (Figure 4.13), cells apparently not exposed to UV radiation showed some cytotoxicity in response to mock treatment with 73% of cells surviving the mock treatment (Figure 6.12). The transfection of the negative control had no effect on the sensitivity of cells to UV irradiation compared with mock transfected cells (Figure 6.12e). The expression of MTMR3 has no effect on the sensitivity of cells to UV induced cytotoxicity at any dose tested (Figure 6.12d). Expression of RBX1, AIFM3 and LIMK2 had no effect at higher doses (100 and 200 Jm⁻²) compared to the negative control (Figure 6.12a-c). A difference was observed at the lowest dose, with 75%, 78% and 84% of RBX1, AIFM3 and LIMK2 expressing cells respectively surviving exposure to 50 Jm⁻² UV radiation, while 64% of negative controls cells survived. However, this difference was replicated in untreated cells, with 86% of RBX1 expressing cells, and 85% of both AIFM3 and LIMK2 expressing cells surviving the mock transfection, while 73% of negative control cells survived. If the supposedly untreated cells are receiving some dose of UV radiation due to leakage in the experimental protocol, this difference could be due a protective effect from the over-expression of the ORFs. However, if the cytotoxicity is due to some other artefact of the experiment, then the difference at 50 Jm⁻² could be due to the same artefact that is causing the difference in mock treated cells. Again, the difficulty in interpreting the results is compounded by the absence of a positive control, which would show if a difference at higher doses of UV would be expected. This means it is not possible to draw any conclusion from these data other than it does not demonstrate a protective effect of expression of the hit ORFs on UV induced cytotoxicity.

6.6 Discussion and conclusions

In previous chapters an assay for the effect of gene perturbation on sensitivity to TRAIL-induced apoptosis was established. This assay was used to compare methods for siRNA-mediated screening and two RNAi screens assessing the effect of gene knockdown on sensitivity of cells to TRAIL were executed. In this chapter the assay developed was applied to an overexpression screen of 288 high-quality full length ORFs from chromosome 22. As with the RNAi screen of kinases and phosphatase, the screen presented here serves two functions. Firstly it serves as a gene discovery experiment. Secondly it serves as an

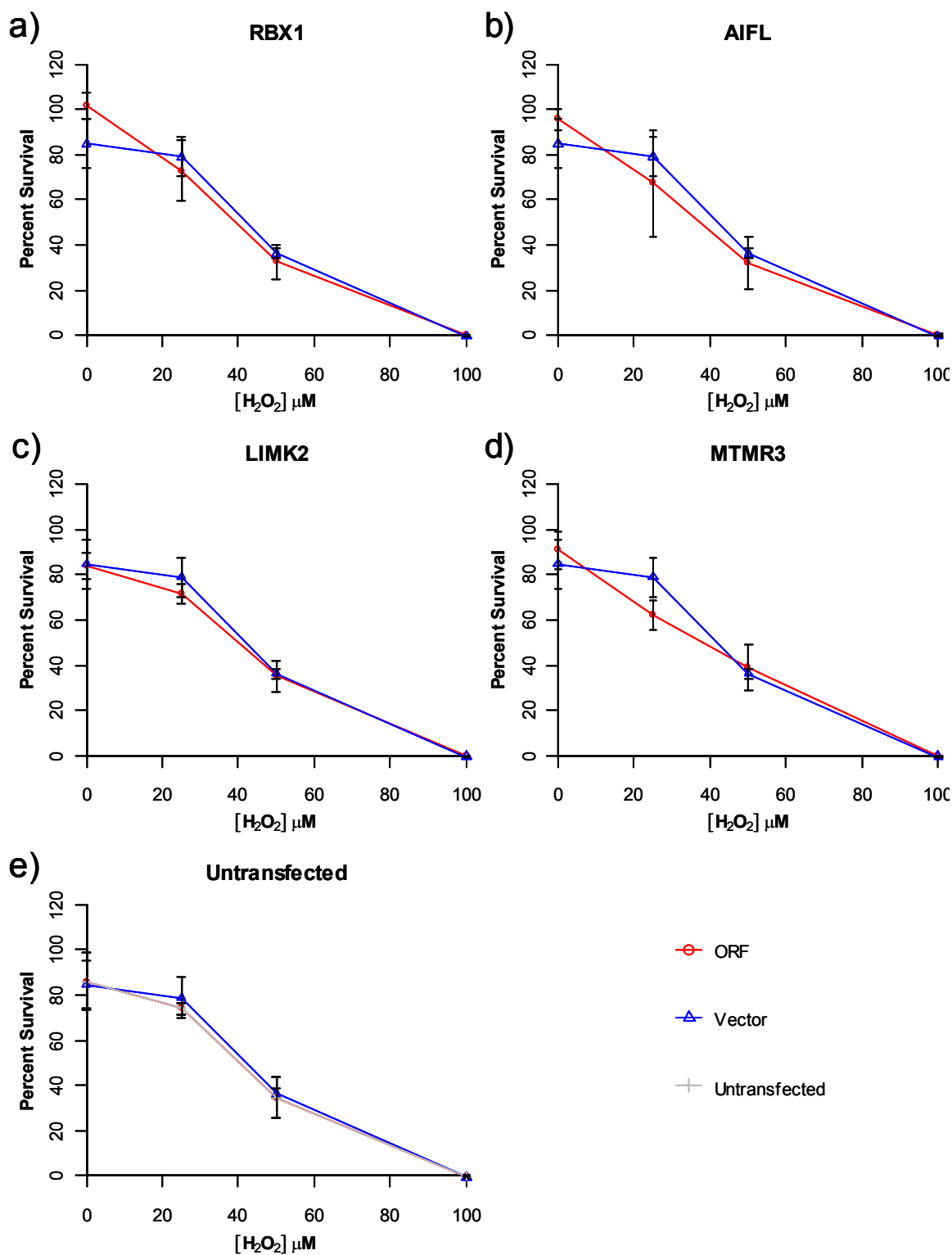


Figure 6.11 Effect of expression of hit ORFs on sensitivity of H₂O₂ induced cytotoxicity

HeLa cells were transfected with constructs expressing a) RBX1, b) AIFM3, c) LIMK2, or d) MTMR3 ORFs, an empty pCDNA3.T7 vector, or e) mock transfected. 48 hours after transfection viability of cells was assessed and cells were treated with the concentration of H₂O₂ indicated. Viability was reassessed 24 hours later. After a further 24 hours viability was reassessed. Error bars represent 1 standard deviation, n = 3.

investigation of the usefulness of such gene-by-gene ORF overexpression experiments.

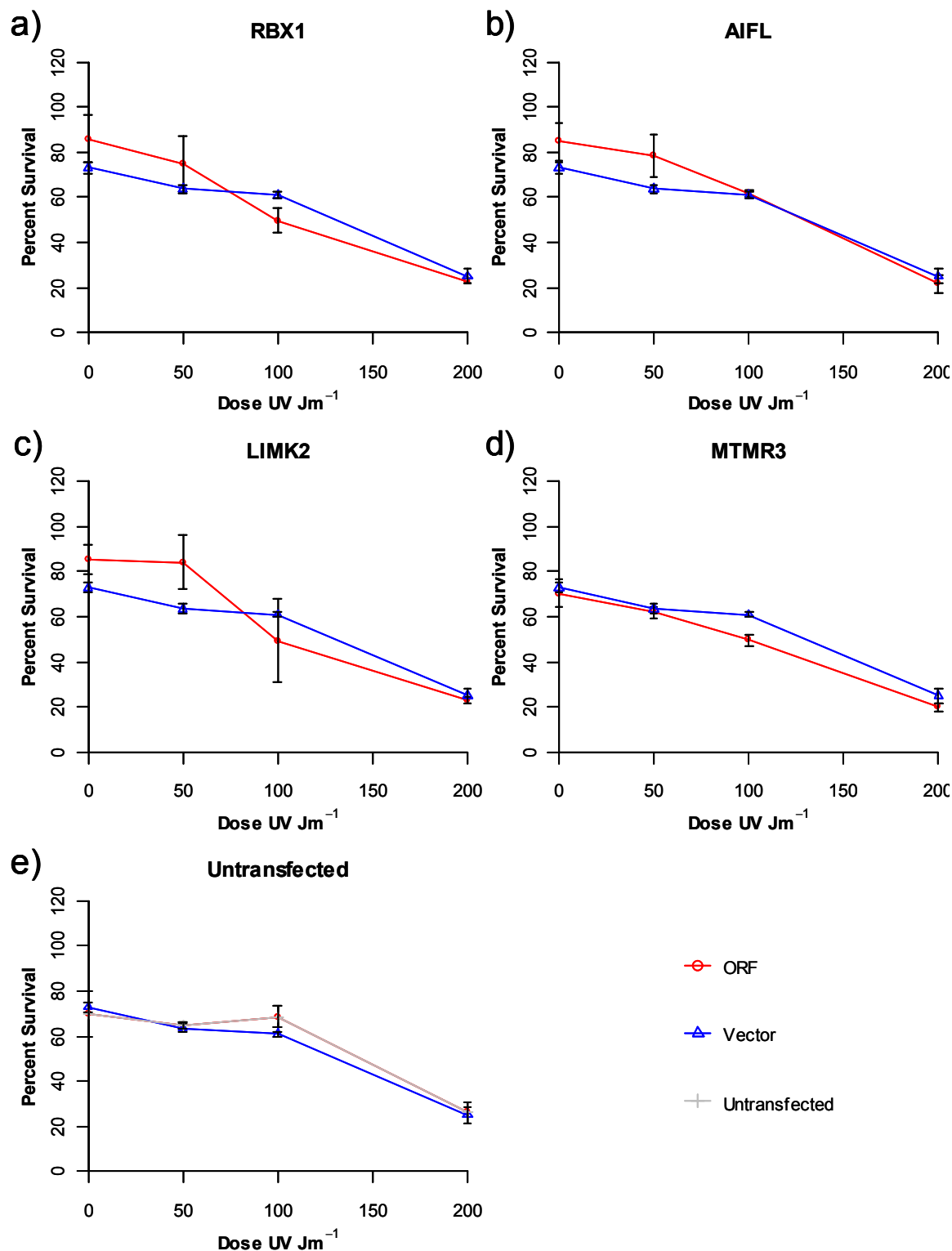


Figure 6.12 Effects of expression of hit ORFs on sensitivity of cells to UV irradiation

HeLa cells were transfected with constructs expressing the a) RBX1, b) AIFM3, c) LIMK2 or d) MTMR3 open reading frames, the empty vector pCDNA3.T7 or e) mock transfected. 48 hours after transfection viability of cells was assessed and cell were exposed to the dose of UV irradiation indicated. Viability was reassessed 24 hours later. Error bars represent 1 standard deviation, n=3

An expression screen of 288 ORFs from chromosome 22 was undertaken to identify

ORFs that could protect cells from TRAIL-induced apoptosis. Four constructs were identified from the screen whose effects clearly deviated from the distribution of effects for the majority of the constructs (Figure 6.6b). These four constructs expressed four different ORFs. When transferred to an untagged expression vector, expression of three of these four ORFs continued to show a significant effect on TRAIL-induced cytotoxicity (Figure 6.7), although only in two cases was this difference still significant when differences in the viabilities of untreated samples were taken into consideration. It was also shown that transfection with these constructs did lead to an increase in transcript levels (Figure 6.9).

Experiments examining the role of these genes in apoptosis failed to show any effect of expression of these ORFs on TRAIL-induced caspase activity or any effect on cytotoxicity caused by the non-ligand apoptosis induction by H₂O₂ or UV irradiation. However, the lack of appropriate controls makes the results of these experiments difficult to interpret. Without a positive control, it is impossible to say if the assays were sensitive enough to detect a change any chance in sensitivity to these apoptosis inducing conditions. Further, had a difference been detected, it would have been difficult to determine if the results were purely due to the over-expression of protein, since the negative control did not express any protein, although possibly in this situation MTMR3, which showed no activity in the TRAIL assay, could have served as a control for this.

6.6.1 The screen

In the previously described siRNA screens a correlation between the rank of the mean normalized survival for an siRNA and the standard deviation between the replicates was observed. The relationship between the rank of the mean normalised survival for an overexpression construct and the standard deviation between the replicates was examined. It was observed that constructs with a higher mean normalised survival tended to have a high standard deviation. Log transformation of the data reduced the strength of this relationship (Figure 6.3). This was supported by the finding that the majority of the scores from the screen calculated using log transformed data are normally distributed (Figure 6.6b).

The normalised survival observed for any given data point is affected by two factors. The first is the biological effect of overexpression of the ORF expressed by the construct transfected into the cells. The second is the random variation in the system. The low correlation seen between replicates of the same construct suggest that in the majority of cases here the random variation in the system dominates the biological effect of ORF expression (Figure 6.5a). The similarly low correlation between constructs expressing the

same ORF tagged at opposite termini could be due to differing biological effects of expressing the ORF tagged at one terminus compared to the other (Figure 6.5b). For example, the T7 tag could interfere with localization signals when fused to one terminus, leading to mis-localization of the protein. Alternatively, the bulk of the tag could interfere with protein folding. However, given the lack of correlation between replicates of the same construct, it seems likely that the lack of correlation here is also largely due to random variation. This lack of biological variation could suggest two things. Firstly it could suggest that the level of overexpression of the ORFs caused by transfection with the constructs is insufficient to trigger an effect that is greater than the level of random variation. In this way, the screening method outlined here would share the same problems as the shRNA screening method explored previously: a low/variable transfection efficiency and insufficient transcription from transfected constructs. Alternatively, it could be due to a genuine lack of ORFs which, when over-expressed, cause a reduction in sensitivity to TRAIL-induced apoptosis. Unfortunately in the absence of multiple repeats of negative and positive controls separate the random variation from the biological variation. Given that a TRAIL sensitive cell has a complete and functional pathway, it is necessarily the case that there will exist genes which when knocked down will disable this pathway. While genes may exist, which when overexpressed, actively inhibit the pathway, the existence of such gene is not necessary. Therefore, an idealised knock-down screen can always be expected to identify genes involved in the pathway, while this is not necessarily the case for an overexpression screen.

Given the conclusion that the random variation is dominating the biological variation, it is logical to assume that the largely normal distribution of scores resulting from the analysis of screening data represents random rather than biologically relevant variation (Figure 6.6a). The normality of this distribution supports the use of log transformation in analysis of survival values. The hits selected for confirmation are found outside this normal distribution, suggesting that the higher survival observed is due to biological, as well as random variation (Figure 6.6b). This conclusion is supported by the finding that three of the four ORFs selected for confirmation showed a significant effect on TRAIL sensitivity when expressed without a tag. The one clone that did not show a significant effect on TRAIL sensitivity was the lowest scoring of the four ORFs selected for confirmation (Table 6-1). However, since these results were not compared to the results of retesting a random selection of genes, it is not possible to definitively conclude that the screen performed better than random for selecting genes, the overexpression of which has an effect on TRAIL-induced cytotoxicity.

It was shown that transfection of constructs expressing ORFs identified in the screen led to an increase in ORF transcript levels (Figure 6.9) and that in three of the four cases transfection lead to an increased survival after treatment with TRAIL (Figure 6.7) compared to empty vector or a ORF which did not score highly in the screen, although in at least one case it is possible that this difference is due to a increase in cell viability in the absence of TRAIL. However, the biological significance of this finding is unclear. The experiments aimed at characterising the ORFs identified as hits from the screen and whose effect was confirmed in the following experiments failed to show that overexpression of these ORFs changed the activity of any of the caspases (Figure 6.10) or that expression of these ORFs altered the sensitivity of cells to any of the non-ligand inducers of apoptosis tested (Figure 6.11 and Figure 6.12). It is important to note that this lack of evidence for involvement of hit ORFs in regulating caspase activity or non-ligand induced cytotoxicity is not evidence for the lack of involvement, particularly given the lack of good controls. Such a situation was also observed when characterising the effect of siRNAs targeting Sharpin and MAST4. In each case one of the two siRNAs targeting these genes could be shown to have an effect on the sensitivity of cells to TRAIL, but not on caspase activity. In each case the siRNA inducing the smaller change in sensitivity to TRAIL-induced cytotoxicity was the siRNA which failed to induce a difference in TRAIL-induced caspase activity. One possible explanation is simply that the assays for caspase activity and assays for the effect of non-ligand apoptosis inducers are less sensitive than the assay for effects on TRAIL-induced cytotoxicity. This seems unlikely in the case of measurements of caspase activity, as transfection of pSM2.shCasp8.2 had a similar or smaller effect on TRAIL-induced cytotoxicity, but a significant effect on caspase activity. The lack of a positive control in the measurement of sensitivity to non-ligand inducers makes it difficult to draw conclusions as to the sensitivity of the assays measuring the sensitivity of cells under these conditions.

Caspase-dependent apoptosis is only one of several forms of programmed cell death, with others including caspase-independent apoptosis, autophagy and programmed necrosis (Reviewed: Assuncao Guimaraes, Linden 2004). There are several reports of TRAIL triggering caspase-independent cell death (Holler et al. 2000, Thon et al. 2006). It is possible that the ORFs identified in this screen are affecting some aspect of this caspase-independent cell death pathway. Alternatively these genes could be involved in the apoptotic pathway downstream of Caspase-3. In both cases it seems unlikely that all three genes identified in a screen should affect sensitivity to TRAIL in this way.

The final possibility is that the finding that overexpression of these ORFs reduces

sensitivity of cells to TRAIL-induced cytotoxicity has no biological significance in terms of the natural functioning of the TRAIL-induced apoptosis pathway. The result could be merely an artefact of the screening system, for example, simply due to the amount of overexpression and the unpredictable effects this has on the function of the cell. Indeed all overexpression studies suffer from similar problem. That a reduction in the level of some gene in a network affects the output of this network gives an indication of the natural function of the network. However, an effect on the network output of introducing a novel factor into the network does not necessarily say anything about the natural function of the network.

6.6.2 The Hits

The above discussion notwithstanding, three ORFs were identified that when overexpressed, did lead to a reduction of the sensitivity of cells to TRAIL-induced cytotoxicity (although possibly not TRAIL-induced caspase-dependent apoptosis).

6.6.2.1 RBX1

RBX1 is a RING-finger protein and member of the SCF E3 ubiquitin ligase complex (Ohta et al. 1999). Ubiquitin ligase complexes catalyse the addition of ubiquitin to their targets, marking them for degradation by the proteasome. Proteasome inhibitors are known to sensitise resistant cells to TRAIL-mediated apoptosis (Ganten et al. 2005). RBX1 has been shown to catalyse the ubiquitination of I κ B, which would lead to an activation of NF- κ B (Ohta et al. 1999). RBX1 has also been shown to bind procaspase-3 leading to its ubiquitination and degradation. Overexpression of RBX1 leads to a reduction of the steady state levels of procaspase-3 and its knock-down leads to a sensitization of cell to TRAIL-induced apoptosis (Tan et al. 2006).

If overexpression of RBX1 protects against TRAIL-induced apoptosis, as shown here (Figure 6.7), via the increasing the ubiquitination of pro-caspase-3 and therefore reducing its levels, it is unclear why no decrease in TRAIL-induced Caspase-3 activity was observed, and why RBX1 overexpression did not offer protection against non-ligand inducers of apoptosis as no change in Caspase-3/7 activity was seen (Figure 6.10, Figure 6.11 and Figure 6.12). If this is indeed the case it suggests a defect in the follow up experiments, despite the fact that the positive control showed an effect. Possibly this suggests that the positive control used in the caspase activity experiments was unsuitable. Despite these discrepancies the finding that one of the genes identified in the screen has previously been associated with TRAIL-induced apoptosis lends confidence to the idea that ORF

overexpression screening can be used to identify genes involved in TRAIL-induced apoptosis.

6.6.2.2 AIFM3

The AIFM3 gene encodes the Apoptosis Inducing Factor; Mitochondrial associated 3 protein (also known as AIF like). AIF is released from the mitochondria along with cytochrome c and DIABLO upon death signalling. Overexpression of AIF triggers a caspase-independent form of apoptosis (Joza et al. 2001, Moubarak et al.). However, the role of AIF in apoptosis control is more complicated, since in some cell types down regulation of AIF also sensitizes cells to apoptosis induction by cellular stress, but not apoptosis inducing ligands. The protection afforded to cells by AIF is dependent on the pyr_redox domain of the protein, which is responsible for its reactive oxygen species (ROS) generating NADH oxidase function (Urbano et al. 2005). AIFM3 is 35% similar to AIF with that similarity mostly residing in the region homologous to the pyr_redox domain. Overexpression of AIFM3 has been shown to induce apoptosis in a cytochrome c and Caspase-3 dependent manner. However, this apoptosis induction was dependent on the Riseke domain of the protein, which isn't found in AIF (Xie et al. 2005). No anti-apoptosis role for AIFM3 has been reported, although since it contains the domain of AIF which is responsible for that protein's anti-apoptosis activity, it is possible that AIFM3 also has both pro- and anti-apoptotic activity.

6.6.2.3 LIMK2

LIM kinases are a family of kinases which regulate actin cytoskeleton dynamics in response to several stimuli, particularly the Rho effector kinase ROCK (Reviewed: Scott, Olson 2007). Other functions for LIMK have been demonstrated including LIMK2 activation of cyclin A1 (Croft, Olson 2006) and involvement in the spindle assembly checkpoint (Sumi et al. 2006). Overexpression of LIMK2 has been reported to lead to membrane blebbing reminiscent of that seen in apoptosis (Amano et al. 2001). LIMK1 but not LIMK2 contains a Caspase-3 target sequence and has been reported to be a target of Caspase-3 (Tomiyoshi et al. 2004), but there are no reports of either LIMK being involved in regulation of apoptosis.

Examination of the expression patterns reported by the GNF expression atlas does not support a correlation between LIMK2 and TRAIL sensitivity. The two cell lines from the NC160 panel of cell lines showing the strongest expression of LIMK2 are COLO205 and

SK-MEL-28 (<http://symatlas.gnf.org>) , which are respectively very sensitive and insensitive to TRAIL-induced apoptosis (Bae et al. 2007, Lippa et al. 2007).

6.6.3 Conclusions

The aims of this screen were two-fold, firstly to identify novel genes involved in the TRAIL-induced apoptosis, and secondly to assess the general usefulness of the approach taken to identifying genes involved in biological pathways.

Constructs, representing each of the 288 ORFs fused to a T7 epitope at both the C and N terminals, were transfected into cells in duplicate and the sensitivity of transfected cells to TRAIL measured. The data was normalised to the median of each plate, log transformed and standardized. The minimum of the two repeats was taken as the score for each construct.

A low correlation between the normalized survivals of the two replicates demonstrates that in the majority of cases the random variation in the system is greater than any biological effect. This shows that either overexpression of most ORFs has no biological effect, or that the system is not sensitive enough to pick any genuine biological signal present. This is in contrast to the results from RNAi screens where a strong correlation exists between the two replicates performed, suggesting that many siRNAs have a varying degree of influence on the sensitivity of the cell to TRAIL-induced cytotoxicity, although this could simply be a measure of the number and strength of off-target effects elicited by the siRNA in question.

Transfection of 4 constructs caused an effect which was significantly outside the distribution of effects of the other constructs, although in the absence of a positive control in the screen it is impossible to comment on the size of these effects. The ORFs from these constructs were transferred to an expression vector which did not fuse a T7 epitope to the ORF. Transfection of three of these four constructs led to a significant reduction in sensitivity to TRAIL-induced cytotoxicity, although in one case this was not significant when pre-treatment viability was taken into account. However, the biological interpretation of these results is difficult since transfection of these constructs did not change the level of TRAIL-induced caspase activation for any of the caspases. Transfection of none of these constructs altered the sensitivity to the non-ligand apoptosis inducers H₂O₂ and UV radiation. It is unclear in these cases whether the characterisation experiments are faulty or if the original results were an artefact of the assay with no biological significance, or if the

ORFs identified are involved in the some pathway parallel to the caspase-dependent apoptosis pathway or act down-stream of the caspase cascade. This is particularly true given the lack of good controls in these experiments.

One of the genes identified, RBX1, a member of the ubiquitin ligase complex SCF, has been previously shown to be involved in the regulation apoptosis through regulation of the stability, and therefore steady state level of, Caspase-3. This shows that the screening paradigm presented here is capable of identifying true regulators of the apoptosis pathways. This finding suggests that the characterisation experiments were not sensitive enough to capture the role of this gene in the regulation of Caspase-3 activity or apoptosis inducing agents other than TRAIL. The second gene, AIFM3, is a member of the apoptosis inducing factor family. AIF has been shown to be have both pro and anti-apoptotic activity. Over-expression to AIFM3 has been shown to induce apoptosis, but is homologous to AIF in the domain that is responsible for AIFs anti-apoptotic activity. The third gene, LIMK2, is a kinase involved in controlling remodelling of the actin cytoskeleton. It has no previously reported affect on regulation of apoptosis. That a plausible story can be constructed for the involvement of two of the three hit genes suggests that the system maybe capable of identifying genes involved in the process. However, the construction of a plausible story for the involvement of these genes does not constitute evidence of their involvement.

Taken together these results suggest that ORF-by-ORF over-expression screening can identify ORFs that affect TRAIL-induced cytotoxicity, although the level of noise is high and it is impossible to say if the identification of hit is better than selecting genes at random. . They show that interpretation of the biological significance of these results can be difficult, including whether these hits are involved in TRAIL-induced apoptosis, or simply TRAIL-induced cytotoxicity. Even if a role in the pathway may be defined for the overexpressed ORFs *in vitro*, it is unclear whether this role has any relevance to the pathway *in vivo*

7 DISCUSSION AND CONCLUSIONS

One of the great challenges for geneticists and molecular biologists is to turn the wealth of sequence data generated by genome sequencing projects into medically relevant functional knowledge. A large part of this is the definition of the function of the many protein coding open reading frames identified in genomes. Observational techniques such as expression profiling and interaction studies can imply a wealth of information about associations of open reading frames and allow the generation of hypotheses on the basis of these associations. However, direct intervention in the functioning of a gene has long been the geneticist's weapon of choice for defining function in the context of a particular pathway

or process. The availability of genome sequence and the development of high-throughput gene perturbation techniques in mammalian cell culture finally allows the extension of this work into a human system. Using the techniques of RNAi and ORF expression, pathways directly relevant to human disease can now be investigated.

The dream of many a cancer biologist is to turn some property uniquely inherent in the nature of the cancer cell against it. Initially, sensitivity to TRAIL-induced apoptosis seemed to be just such a property, with transformed, but not non-transformed cells being sensitive to apoptosis triggered by TRAIL (Walczak et al. 1999). However, it was soon found that some cancer cell types were resistance to TRAIL-induced apoptosis (Zhang et al. 1999). The mechanisms which distinguish normal cells from transformed cells and sensitive from insensitive transformed cells are not fully understood.

Thus the aims of this work were two fold. Firstly to examine, assess and compare different methods for genome scale gene perturbation and secondly to apply these methods to the identification of novel genes involved in the regulation of sensitivity to TRAIL-induced apoptosis.

The success of screening experiments depends critically on careful selection of assay conditions and selection of gene perturbation technique. Plasmid based hairpin RNAs present an attractive technique for gene knock-down. Such plasmids are an infinite resource, allow for selection of transfectants/transformants, allow introduction into hard-to-transfect cell types, and critically allow the execution of pooled selection type screens. However, the experiments in Chapter 3 suggest that the shRNAs containing within the shRNA library to which the author had access do not elicit phenotypes as strong as those elicited by chemically synthesised siRNAs. One explanation of this is that siRNAs are easier to transfect than shRNAs, reaching higher transfection efficiencies. However, the gap between shRNAs and siRNAs is not closed by using drug selection to increase transfection efficiency, which has more of an effect on the sensitivity of cells transfected with the negative control than it does on cells transfected with an shRNA targeted at knocking down a gene of interest. There is little data in the literature directly comparing shRNAs with siRNAs. The original report of shRNAs functioning in mammalian cell culture demonstrated that for a single sequence targeting Luciferase shRNAs were as effective as siRNAs in a dual Luciferase assay that would normalise for expression from the plasmids (Paddison et al. 2002). It has been reported that chemically synthesised hairpin RNAs outperform chemically synthesised siRNAs. However, the authors did not test for induction of the interferon response, which is a possibility with RNAs of that length (Siolas et al. 2005). One possibility for the difference

between the shRNAs and siRNAs tested here is that the sequences of the shRNA were designed according using different algorithm to the siRNA sequences. Once designed and created, the sequences of shRNAs are more likely to stay stable, while for siRNAs, sequences can be updated as the siRNAs are re-synthesised. This means that siRNAs purchased are more likely to have been designed using more recent algorithms than shRNAs, and therefore are likely to be more effective. Further, with the decreasing price of siRNA synthesis and taking into account the cost of preparing plasmid DNA to transfection quality for the many thousands of constructs required for a screen, the price advantage of shRNAs in terms of reagent cost is less clear. However, the cost of the RNAi library is a fraction of the total cost of conducting a screen, with the total cost of plasticware, cell culture media, transfection reagent and assay materials easily totalling more than the cost of the library. By dramatically reducing the number of experiments necessary to conduct a screen, pool selections have the potential to reduce the total cost of a study. This is where shRNAs could be a real advantage. However, such an approach relies on the assay in question having a very high signal/noise ratio, and therefore a powerful, reliable method for reducing gene expression. Pooled screening also reduces the range of processes that can be studied. Although there are several examples of successful pooled selections using shRNAs (Berns et al. 2004, Hattori et al. 2007, Kolfschoten et al. 2005, Nicke et al. 2005, Paradis et al. 2007, Westbrook et al. 2005), siRNAs will remain the reagent of choice, particularly for those phenotypes where the difference between hits and negatives is quantitative rather than qualitative.

Although at high-throughput there is obviously a large amount of variance in the results of a quantitative assay, the results for particular siRNAs are fairly reliable. This does not, however, translate into a high reliability in the results for particular genes. There are two possible reasons for this. Firstly variance in the efficiency of an siRNA in knocking down the intended target, and secondly a result for an siRNA can be due to off-target effects, rather than due to the effect of knocking down the intended target (or a combination of both). The process of screening is likely to exacerbate both problems. The screening process selects siRNAs which have a large effect on the assay. Therefore, the highest scoring siRNAs are likely to be the ones that have the largest effect on transcripts which affect the assay. These transcripts may be the intended target or other transcripts which affect the assay. It is therefore not surprising that other siRNAs targeting the same transcript are often not as efficient at knocking down the transcript of interest. It is possible that this problem could be reduced by more efficient introduction of siRNAs into the cell. Indeed, conditions in the assay here were not optimised for maximal transcript knockdown, but rather for largest

effect on the assay, which is a combination of conditions that allow knock-down of the transcript in question and the conditions that give maximal sensitivity to TRAIL.

The other problem with using a protocol designed to introduce more siRNA into the cell is that this will increase the severity of phenotypes caused by off-target effects. The off-target effects are generally weaker than the on-target effects (Birmingham et al. 2006, Jackson et al. 2003). Thus the ideal level of knock-down is one where the reduction of the intended target is sufficient to elicit a phenotype, but the level of knock-down of off-target transcripts is not. It is often assumed that the finding that a phenotype induced by an siRNA is due to off-target effects is unlucky – the exception rather than the rule. This may be the case for single gene experiments, where siRNAs targeting a gene are chosen on an arbitrary basis rather than on the basis of their performance in an assay against many thousands of others. However, the work here suggests that in the screening situation it is more likely that at least part of the effect of an siRNA is due to its off-target effects than in single gene experiments. Analysis of seed sequences in the high scoring siRNAs such as that performed here or in (Lin et al. 2007) may help to identify suspect seeds and siRNAs containing them. However, it is clear that the seed sequences of an siRNA does not wholly determine its activity in an assay. While other determinants of siRNA off-targeting have been described (Nielsen et al. 2007), it seems likely that the effects of any siRNA are the combination of a large number of weak effects on off-target transcripts and a single, large effect on the intended target.

These conclusions support several practical suggestions for following up RNAi screens. Firstly, a hit should be confirmed with multiple siRNAs, preferably more than two, and further, these siRNAs should not include any siRNAs included in the screen. siRNAs used for confirmation should also be checked to ensure that they do not contain seed sequences that are enriched in high-scoring siRNAs from the screen. It has been reported that chemical modification of siRNAs can increase their specificity. The addition of various groups to the nucleotides on the passenger strand of the siRNA prohibits its entry into RISC, and the addition of an O-Methyl group to base 2 of the guide strand may also reduce the number of off-target effects triggered by this strand (Jackson et al. 2006). See the introduction for further details. The use of siRNAs which have been chemically modified could be of use here, particularly if the chemically modified siRNAs with the same sequences as those from the screen failed to elicit the same phenotype. Finally, if at all possible hits should be confirmed using rescue or inhibition through some non-RNAi method. This final suggestion is often repeated (Echeverri et al. 2006, Sarov, Stewart 2005) and rarely followed, due to the difficulty associated with such experiments. However, there are examples of RNAi

results being confirmed by rescue using either expression of an ORF to rescue siRNAs targeted at the 3' UTR of a gene (Yi et al. 2007) or by using transfection of a mouse BAC to rescue an siRNA directed at a human gene (Kittler et al. 2005).

RNAi screening is often thought of as a “reverse genetics” technique: each gene is methodically tested in turn and its effect on the process of interest recorded. However, RNAi screens are far from 100% sensitive. The overlap between the screen of kinases and phosphatases presented here and another screen of the kinome is minimal (Aza-Blanc et al. 2003), and the druggable genome screen failed to have several highly important genes amongst its top hits: while, for example, library siRNAs targeting Caspase 8 may have scored highly in the screen, siRNAs targeting PDE11A, a gene which is surely less important in the TRAIL-induced apoptosis pathway, scored more highly. This, taken together with the fact that results from an siRNA cannot be guaranteed to be solely related to the intended target, suggest that RNAi screening is analogous more to traditional or forward genetics. Instead of providing a quantitative readout of the involvement of each and every gene in a process, they can identify a set of genes with a confirmed qualitative effect. As such RNAi screens are powerful tools, which can identify novel genes involved in a pathway, but should not be regarded as “saturating”. This is also the case for RNAi screens in model organisms. A genome wide screen of the *C. elegans* genome for embryonic and post embryonic phenotypes found identified 63% genes with a known phenotype (Kamath et al. 2003). This is similar to the proportion of genes previously associated with TRAIL-induced apoptosis that could be confirmed in assay development experiments here, although higher than the number of such genes that performed well in the screens themselves. The situation is even more pronounced in model organism cell culture systems. Two independent screens for regulators of the JAK/STAT signalling pathway found 91 and 121 hits respectively. The overlap between these two sets of hits was only six genes (Baeg, Zhou & Perrimon 2005, Müller et al. 2005)

This is highlighted by the results of an siRNA screen for TRAIL regulators published in the last few days of preparation of this thesis, during the composing of this discussion (Ovcharenko et al. 2007). In this study a fluorescent caspase-3 activity assay, whereby cleavage of a caspase-3 substrate leads to an increase in fluorescence was used as a primary assay. The screen included 3 siRNAs targeting each gene, and the screen was performed in triplicate. Caspase-3 activity levels were not normalised to the pre-treatment viability of the cells, so differences in Caspase-3 activity levels could be due to difference in cell growth. Genes were selected as candidate hits if the average score of two siRNAs were above an arbitrary threshold. The presence or absence of positive controls is not reported, results from

Screen	Gene	Score		Average Survival		Rank	
		siRNA A	siRNA B	siRNA A	siRNA B	siRNA A	siRNA B
Druggable Genome	PTGS1	-0.01	-3.50	86.5%	9.0%	4514	10851
	CLCN3	-0.063	-1.07	29.4%	24.0%	7013	8290
Kinase and Phosphatase	CDK2	1.1	0.99	45.0%	30.1%	167	197
	CDK4	0.13	-0.56	37.3%	9.60%	586	1117
	CDK9	-0.33	-0.78	12.6%	13.8%	950	1315
	IRAK4	0.31	-0.15	21.20%	14.30%	466	804
	MAP3K6	-0.62	-0.83	9.8%	14.9%	1261	1459
	MAP3K8	0.67	0.52	28.70%	30.90%	294	382
	PAK2	0.27	-0.41	22.5%	10.6%	487	996
	PAK1	-0.74	-1.00	13.3%	8.6%	1273	1502
	HK1	-1.24	-1.45	1.6%	0%	1679	1783

Table 7-1 Performance of siRNAs targeting hits from Ovcharenko *et al* screen in screens performed here

The score, average raw survival and rank in the complete ranking is shown for siRNAs targeting hits from the Ovcharenko *et al* screen that were included in the screens reported in this work. Rank represent position out of 11162 siRNAs for the druggable genome screen and 1785 siRNAs for the kinase and phosphatase screen. The genes ALG2 and LRRFIP1 are reported as hits by Ovcharenko are not included in the library used in this work.

negative controls are also not reported but are used to normalise data. Candidates were validated by performing the same assay on the same siRNAs and applying a t-test to the results. The authors claim to use a 1% p-value threshold, with no multiple testing corrections, but several of the genes they claim as hits do not make this threshold. Again, the authors do not report results from any positive controls. No measurement of the effect of the siRNAs on the level of transcript or protein is undertaken. No secondary assay is undertaken. The authors report seven known and thirteen novel genes which when knocked down reduced sensitivity to TRAIL, although only 5 of the known and 8 of the novel genes meet their own criteria for a hit. None of these genes overlap with the set of genes reported here, and they confirmed only one of the novel genes in a separate screen of kinases (Aza-Blanc *et al.* 2003). The performance of their hit genes in the screens conducted here is shown in Table 7-1. They do report that in their hands, knock-down of IGF1R reduces sensitivity to TRAIL, but does not meet their criteria for statistical significance. Differences between the two screens include the cell line used (MDA-MB-453 cells mammary carcinoma cells vs. the HeLa cervical carcinoma cells used here and in Aza-Blanc *et al*) and the assay used (TRAIL-induced Caspase-3 activity vs. alamarBlue survival assay used here and in Aza-Blanc *et al*). However, it is still significant that none of the genes isolated here were also isolated in this similar screen.

In many ways overexpression screens suffer from the same technical reliability vs. biological reliability issue as RNAi screens. The effects of 3 of the 4 hits from the

overexpression screen tested were confirmed to have an effect on post TRAIL treatment viability (although in at least one case this effect disappears when the effect of overexpression on un-treated cells is accounted for). However, the difficulty in the interpretation of the biological significance of these hits is not connected with associating the technical results with the gene being overexpressed, but what it means, biologically, that overexpressing these genes leads to this phenotype. Although the hits from the overexpression screen of chromosome 22 genes could be shown to reliably affect TRAIL-induced cytotoxicity, no effect on TRAIL-induced caspase activity, or sensitivity to other apoptosis inducers could be shown. This could be due to a question of sensitivity, as one of the hits RBX1, had previously been shown to have an effect on levels of Caspase-3 (Tan et al. 2006). Indeed, that two of the three hits could be connected to regulation of apoptosis suggests that overexpression screening can provide relevant results. Even so, it is unclear what the true meaning of an overexpression phenotype is. If a protein is expressed at a higher level than is found in an *in vivo* setting or in an environment which it is not normally expressed, is its effect necessarily indicative of its *in vivo* function? Many of the early experiments which suggested that the TRAIL decoy receptors were involved in regulation of TRAIL sensitivity used overexpression of these receptors (Degli-Esposti et al. 1997a, Degli-Esposti et al. 1997b, Emery et al. 1998, Pan et al. 1997a). Later studies found minimal correlation between decoy receptor expression and sensitivity to TRAIL-induced apoptosis (Ganten et al. 2004, Kim et al. 2000, Zhang et al. 1999).

In the case of the overexpression screen there was a clear distinction between those ORFs that did cause a change in TRAIL-induced cytotoxicity (hits) and those that did not. This distinction was less clear in the case of the RNAi screens, with each siRNA having a quantitative effect on the percentage of cells which survived treatment with TRAIL. It is unclear whether this is due to variability in the efficiency of the siRNAs knocking down their targets, due to the different complement of off-target effects caused by each of the siRNAs, or whether it is due to differing effects of knocking-down each gene on the sensitivity of cell to TRAIL-induced apoptosis. Indeed, Friedman and Perrimon have suggested that the sorts of continuous distributions seen here in the screen results imply a network model of signalling, where each phenotypic output is not the result of a defined number of genes, but each gene has a smaller or larger contribution to signalling of the network (Friedman, Perrimon 2007). They also argue that such distributions are unlikely to be the product of off-target effects since they are also observed in genetic screens with a quantitative output. The hits identified in the screens here act in seemingly disparate pathways, with members of the

MYC, NF- κ B (IKBKE), tyrosine kinase (IGF1R) and cAMP (PDE11A) pathways identified as well as genes seemingly connected to none of the canonical signalling pathways. Indeed, an RNAi screen for genes involved in regulating Caspase-3 activity in *Drosophila* cells has recently implicated genes encoding metabolic enzymes in the regulation of sensitivity to apoptosis inducing stimuli (Yi et al. 2007). This suggests that rather than the sensitivity of cells TRAIL being the output of a single linear signalling pathway it rather depends on the complex interactions between many of the cell's signalling modules.

Several of these modules are known to have both pro- and anti-apoptotic effects. In many cases, where a gene was isolated here with a known role in control of apoptosis, the role is either anti-apoptotic with the role described here being pro-apoptotic (e.g. TEGT); pro-apoptotic with the role described here being anti-apoptotic (e.g. AIFM3); or both pro- and anti-apoptotic (e.g. IGF1R or IKBKE). IGF1R signals through AKT, which can have both positive and negative effects on TRAIL-induced apoptosis (Chen et al. 2001, Pugazhenti et al. 2000, Remacle-Bonnet et al. 2005, Thakkar et al. 2001). Therefore the effect of IGF1R signalling must depend on the balance of these signals as determined by other parts of the network. The same reasoning applies to the activity of NF- κ B possibly through the balance of the action of the c-Rel and RelA subunits (Ravi et al. 2001). TRAIL itself has both pro- and anti-apoptotic activity through its action on NF- κ B and so transformation must imply either an inhibition of this pro-survival pathway, a strengthening of the pro-apoptotic pathway, or a change in balance of NF- κ B outputs such that NF- κ B activity becomes pro-apoptotic. This all suggests that rather than there being a single mechanism through which cells become sensitive to TRAIL on transformation, any of the alterations to the cells signalling network which led to transformation also lead to a promotion of TRAIL-induced apoptosis.

Given that there may be multiple routes to TRAIL resistance/sensitivity it will be of interest to determine if the same genes are involved in regulation of TRAIL-induced apoptosis in cell lines other than the HeLa cells used here. That is, do the same perturbations of the signalling networks in one cell line lead to the same changes in phenotypic output as in another cell line. One interpretation of the lack of overlap between the screens reported here and a screen for regulators in a breast carcinoma cell line suggests that this isn't the case, although another interpretation is that RNAi screens are not saturating (Ovcharenko et al. 2007).

One overlooked aspect of RNAi screening is that although RNAi screening is analogous to screening hypomorphic mutations in protein coding genes, it is also analogous

to overexpression screening of miRNAs. miRNAs are key regulators of many aspects of cell biology and can control the expression of many genes co-ordinately. It is not unreasonable to suppose therefore that miRNAs are involved in the sensitivity of cells to apoptosis. Indeed, a report published during the preparation of this discussion reports a list of miRNAs, overexpression of which changes the sensitivity of cells to TRAIL-induced Caspase-3 activity (Ovcharenko et al. 2007). Included in this list are miR-145 and miR-155, miRNAs which were identified in chapter 5 as containing seed sequences which were either over-represented in the hit siRNAs, or enriched in high scoring siRNAs in general. This supports the idea that seed analysis can isolate relevant and interesting phenomena. It is also reported that miR-26a alters the level of non-TRAIL-induced Caspase-3 activity. miR-26a contains the seed sequence ACTTGA, which is also found in four of the hit siRNAs, and is enriched in the high-scoring siRNAs in general. It also appears multiple times in the 3' UTRs of DR4, DR5 and BIS. Thus it is possible that siRNAs containing this seed are acting as a miRNA and knocking down the same transcripts as miR-26a. This would also implicate miR-26a in the regulation of Trail-induced cytotoxicity.

7.1 Future directions

In addition to the confirmed hits reported here, each of the siRNA screens identified a number of “unconfirmed” hits. These genes were “targeted by only one siRNA which significantly altered the sensitivity of cells to TRAIL-induced cytotoxicity or Caspase-3/7 activity, further siRNAs did not significantly alter the sensitivity of cells to TRAIL, but neither did they knock-down the targeted transcript to the same extent. Results from further siRNAs could help either to categorise these genes as hits or identify that the effects of the original siRNAs were due to off-target regulation. Results from further siRNAs could also increase confidence in several of the genes here classified as confirmed hits. Sharpin, MAST4, IKBKE, INADL and TEGT are all genes for which questions as to their role in TRAIL-induced apoptosis remain. For Sharpin and MAST4 siRNA targeting them gave contradictory results in caspase activity assays, while siRNAs targeting IKBKE give contradictory results in experiment examining the effects of knockdown on sensitivity to a range of apoptosis inducers. Either one or both siRNAs targeting INADL and TEGT contain suspected seed sequences – confidence would be increased by results from siRNAs not containing these seed sequences.

As stated above, the final confirmation of an siRNA result is given by rescue of the RNAi induced phenotype. This can be achieved using siRNAs directed against the UTR

sequence of the gene in question, and rescuing the phenotype by expressing the gene's open reading frame from a plasmid construct without the UTR sequence. This is made easier by the availability of ORF clones for a large portion of the genome. It will also be of interest to see if the result of knocking down these genes in HeLa cells can be replicated in other cell types.

Ultimately, confirmed hits simply form a list of genes. Our knowledge of a process is only really increased when the role of these genes in the process is understood. Although studying the literature can help to generate hypotheses about how these genes are involved in TRAIL-induced apoptosis, only direct experimentation can confirm or refute these hypotheses. A good place to start in a functional exploration of the effects of these genes is their effect on the expression levels of known direct actors in TRAIL-induced apoptosis. Obvious candidates are the death receptors, the cFLIP DISC inhibitor and Mcl-2, all of which have been previously reported to be the endpoints of pathways regulating TRAIL-induced apoptosis (Wang et al. 2004/5, Ricci et al. 2004, Ricci et al. 2007). In the case of the death receptors, it is important that it is the surface expression rather than the bulk protein or RNA level that is measured (Ren et al. 2004).

Some of the hypotheses for the action of hits suggested by the literature make specific testable predictions. For example, if PDE11A functions to inhibit AKT induced Bcl-2 expression through CREB (Pugazhenthii et al. 2000), then PDE11A knock-down should increase CREB-mediated transcriptional activation, which could be measured using a reporter system.

Little is known about the effects of miRNAs on apoptotic pathways, with the first reports beginning to be published about connections between miRNAs and the TRAIL pathway (Ovcharenko et al. 2007). The involvement in the regulation of TRAIL sensitivity of two of the four miRNAs sharing one of the seed matches, highlighted as suspicious by an analysis of seeds in high scoring siRNAs, has already been confirmed (Ovcharenko et al. 2007). Transfection of artificial miRNA mimics with the same sequence as miR-26a and miR-384 could determine if these miRNAs regulate TRAIL sensitivity in HeLa and other cell lines. It would also be of interest to see if the expression level of these miRNAs in clinical isolates is correlated with the sensitivity of these isolates to TRAIL-induced apoptosis.

Finally, the seed analysis performed in this work is, to the best of the author's knowledge, the most thorough investigation of seed sequences in the hit list of an siRNA screen ever performed. However, while this analysis allows the generation of many hypotheses, it does not prove any of them. Possibly the easiest hypothesis to investigate

would be that siRNAs with seeds from the “enriched” seed set have, on average, higher numbers of off-target effects than other siRNAs. Expression profiles could be generated for cells transfected with siRNAs containing these seeds and compared with expression profiles for cells transfected with siRNAs targeting the same genes which did not contain suspect seed sequences. Indeed, such profiles might also help to identify the off-target effects of import for TRAIL-induced apoptosis. On a smaller scale, the effect of siRNAs containing suspect seed sequences on known regulators of TRAIL-induced cytotoxicity could be studied using qRT-PCR.

Another way in which off-targeted transcripts could be identified, would be to investigate the effect of these siRNAs on genes predicted to be off-targets, and also the effect of intentional knock-down of these predicted targets on TRAIL-induced apoptosis. One method for predicting these targets was outlined in Chapter 5. However, very recently, more sophisticated algorithms for predicting the off-target effects of siRNAs have been suggested. Nielsen *et al* describe an algorithm based on the number and length of seed matches, plus the AU content, and conservation, of the sequence surrounding the matches, which allowed the prediction of the off-target effects of several siRNAs (Nielsen et al. 2007). An implementation of this algorithm could be developed to predict the most common off-target effects between siRNAs containing suspect seeds.

The hypothesis that the effect of siRNAs targeting hit genes from the screen is a combination of off and on target effects could also be tested. Four siRNAs could be designed, two targeting a gene known to be involved in the TRAIL-induced apoptosis pathway (gene A), and two targeting a gene known not to be involved (gene B). One siRNAs targeting each of the genes would contain a suspect seed sequence and one would not. If the siRNAs targeting each gene were of similar efficiencies, the hypothesis would predict that the siRNA targeting gene A which contains the suspect seed would have the largest effect on TRAIL-induced apoptosis, follow by the siRNA targeting gene A which does not contain the suspect seed and the siRNA targeting gene B which does. Finally the siRNA targeting gene B which does not contain the seed would be predicted to have minimal effect.

Several of these suggestions are simple and should not take long to perform, in particular the testing of additional siRNAs against hits, the titration of the amount of siRNA used, and the testing of miRNA mimics. These were not performed due to severe time limitations towards the end of this project. Other suggestions are more open ended, such as avenues of investigation into the biological relevance of the selected hits, and the microarray experiments aimed at further understanding the importance of hit and enriched seeds, both

of which constitute separate projects in their own right. Finally, while it would be possible to repeat the overexpression screen and follow-up experiments using a positive control and better negative controls, the negative nature of the results suggest that this would not be a good use of time and resources.

7.2 Conclusion

This work has shown that, at least for screening in the TRAIL-induced apoptosis system, siRNAs are a more powerful tool for functional screening than shRNAs. It has shown that RNAi screening identifies siRNAs that have a reproducible effect on the process of interest. Although RNAi screening is less reliable at identifying genes than siRNAs which are involved in the process of interest, genes with a confirmed effect on the process can be identified. Six novel genes connected with TRAIL-induced apoptosis were identified in this way, along with three genes with a known effect. These genes were from distinct pathways.

This work has also suggests that overexpression screens of cloned ORFs can also be used to identify constructs with a reproducible effect on the TRAIL-induced cytotoxicity assay, although the biological significance of these hits is unknown. Thus the technique did not prove useful for furthering understanding of the pathway.

Seed analysis has shown that the process of siRNA screening can enrich for siRNAs that contain certain seed sequences. Although not demonstrated, the obvious hypothesis is that these seed sequences specify relevant off-target effects. This seed analysis also identified four miRNAs with a possible role in TRAIL-induced apoptosis. The involvement of two of these was confirmed elsewhere experimentally. This shows that a knock-down by an siRNA should also potentially be regarded as an overexpression of a miRNA.

In conclusion, genome-scale systematic gene perturbation studies are powerful tools for annotation of gene function, and in isolating novel genes in medically relevant pathways, but they must be used with care and an awareness of their possible pitfalls. Care should be taken interpreting the function of a gene based solely on isolation in an overexpression screens. Like RNAi screens in model organisms, siRNA screens in mammalian cells do not isolate all of the genes known to be associated with a process and there can be limited overlap between genes isolated in different screens in the same pathway. It also appears that off-target effects may be more prevalent than may have been appreciated in the past. Application of the recommendations outlined above to future screens will help to increase confidence in the results. It should also be remembered that small RNAs have a natural role in the cell and that transfection of an siRNA could be equivalent to overexpression of a

miRNA. However, awareness of this can allow candidate miRNAs connected with the pathway to be identified and turn this potential problem into an advantage

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9 APPENDICES

Appendix A Sequence of siRNAs Used

Both the RNA sequence of the siRNA guide strand and the sequence of the target in the targeted transcript are provided. siRNAs contain 2nt 3' overhangs in all cases except those acquired from Invitrogen which are blunt ended. Sequence provided includes the overhang. Deoxy bases are

denoted by dN. Sequence of siKIFF11 and siNeg, both from Ambion was not provided.

<i>siRNA Name</i>	<i>Sequence (Guide strand)</i>	<i>Sequence (Target)</i>	<i>Supplier</i>	<i>siRNA Number/ Catalogue No.</i>
ABL2 smartpool	Pool		Dharmacon	M00101-01-0005
QiaNeg	ACGUGACACGUUCGGAGAAUU	AATTCTCCGAACGTGTCACGT	Qiagen	1022076
ROS1 smartpool	Pool		Dharmacon	M-003173-01- 0005
siABL2.1	UAUCUGUUCGCUCCAUUUCUU	AAGAAATGGAGCGAACAGATA	Dharmacon	D-003101-05
siABL2.2	UUAAUAGGAAAUUUGGCUCUU	AAGAGCCAAATTTCTATTAA	Dharmacon	D-003101-06
siABL2.3	UAGACUGUAGGCUUAUUACUU	AAGTAATAAGCCTACAGTCTA	Dharmacon	D-003101-07
siABL2.4	UUAGAGCGAACUUCACUCCUU	AAGGAGTGAAGTTCGCTCTAA	Dharmacon	D-003101-08
siACO1.1	UAGUGUAUCGUUCUUGCCCUG	CAGGGCAAGAACGATACACTA	Qiagen	SI02779945
siACO1.2	UUCAAGUGGGAUCACACCCAU	ATGGGTGTGATCCCCTTGAA	Qiagen	SI02779952
siADORA1.1	UUUAUUAGUCACAUGGGCCUC	GAGGCCCATGTGACTAATAAA	Qiagen	SI02624097
siADORA1.2	AUGAUUACUGAACCUACCCUU	AAGGGTAGGTTTCAGTAATCAT	Qiagen	SI00013846
siADORA1.3	UGACAGGUAUUACACUCCAG	CTGGAGTGTAATTACCTGTCA	Qiagen	SI00013860

<i>siRNA Name</i>	<i>Sequence (Guide strand)</i>	<i>Sequence (Target)</i>	<i>Supplier</i>	<i>siRNA Number/ Catalogue No.</i>
siBID	UCUAAAGCUAAGCUUUUCCdTdT	AAGGAAAAGCUUAGCUUUAGA	Ambion	120776
siCasp3.1	UAU AACUGUUGUCCAGGGAUUUUCC	GGAATATCCCTGGACAACAGTTATA	Invitrogen	HSS101371
siCasp3.2	UCCAGUUAGACUUCUACAACGAUCC	GGATCGTTGTAGAAGTCTAACTGGA	Invitrogen	HSS101372
siCasp3.3	AAAGGACUCAAAUUCUGUUGCCACC	GGTGGCAACAGAATTTGAGTCCTTT	Invitrogen	HSS101373
siCasp8	GGGUCAUGCUCUAUCAGAUdTdT	AAAUCUGAUAGAGCAUGACCC	Ambion	AM16100
siCCNT1.1	UACCUUGAUGACAUGUUCCAA	TTGGAACATGTCATCAAGGTA	Qiagen	SI02625714
siCCNT1.2	UUAUGUGCUAAGUUGCUUGUC	GACAAGCAACTTAGCACATAA	Qiagen	SI00024073
siCCNT1.3	UUGAUAGUCUAUUGUCUGGGU	ACCCAGACAATAGACTATCAA	Qiagen	SI00024080
siDCK.1	UUUAAUUCACUUUGCAACUU	AAGTTGCAAAGTGGAAATTA	Qiagen	SI00604940
siDCK.2	UAACAAAUUAAUUUUAUACCAA	TTGGTATAAATTAATTTGTTA	Qiagen	SI00604947
siDYRK1A.1	UUACAAUGCACAGAAGUCCUG	CAGGACTTCTGTGCATTGTAA	Qiagen	SI02626211
siDYRK1A.2	UUAUGUUUGGCUGGCGACGGU	ACCGTCGCCAGCCAAACATAA	Qiagen	SI03040415
siDYRK1A.3	UUUAAUGGCAACCCAUUCUUG	CAAGAATGGGTTGCCATTA	Qiagen	SI00605234
siETNK1.1	UUUCUAAUCCACCACAUACGG	CCGTATGTGGTGGATTAGAAA	Qiagen	SI02224887

<i>siRNA Name</i>	<i>Sequence (Guide strand)</i>	<i>Sequence (Target)</i>	<i>Supplier</i>	<i>siRNA Number/ Catalogue No.</i>
siETNK1.2	UUCUAAUCCACCACAUACGGA	TCCGTATGTGGTGGATTAGAA	Qiagen	SI02224894
siFBXO11.1	AUUCCGUCUAAUAAUUGGGdTdT	AACCCAAUUAUUAGACGGAAU	Ambion	130848
siFBXO11.2	UGCACCUUUUAUACAACUGCdTdG	CAGCAGUUGUAUAAAGGUGCA	Ambion	130846
siFBXO11.3	AGAAAUGUUCAGCAAUCCdTdT	CAGGAUUUGCUGAACAUUUCU	Ambion	130847
siFLJ12312.1	AAGAACAGCGCAUACAGCCdTdT	AAGGCUGUAUGCGCUGUUCUU	Ambion	125977
siFLJ12312.2	ACAUGACUACAGAGAUCdTdT	AAGGAUCUCUGUUAGUCAUGU	Ambion	125976
siFLJ32312.3	CUCUUACAGAUAGUUGUGdTdT	CACCACAACUAUCUGUAAGAG	Ambion	125978
siGL2	UCGAAGUAUCCGCGUACGdTdT	AACGUACGCGGAAUACUUCGA	Ambion	AM16100
siGPR132.1	UUGAUCUCGAUGGUGACCCAG	CTGGGTCACCATCGAGATCAA	Qiagen	SI00101682
siGPR132.2	UUCAGGAACGAGAAAUUGGUA	TACCAATTTCTCGTTCCTGAA	Qiagen	SI00101696
siGPR132.3	UAGUAGUACCCGGCAAUCCUG	CAGGATTGCCGGGTACTACTA	Qiagen	SI00101703
siGUK1.1	GUCGUUAAUGAUGACCACAdTdC	GAUGUGGUCAUCAUUAACGAC	Ambion	242335
siGUK1.2	GUAGUAAUCUUUGCCGUUCdTdT	GAGAACGGCAAAGAUUACUAC	Ambion	130786
siGUK1.3	GACACGCUGAAGCCAAAGAdTdG	CAUCUUUGGCUUCAGCGUGUC	Ambion	242336

<i>siRNA Name</i>	<i>Sequence (Guide strand)</i>	<i>Sequence (Target)</i>	<i>Supplier</i>	<i>siRNA Number/ Catalogue No.</i>
siHAPIP.1	UUCAAGUACAAAGACAGUCCU	AGGACTGTCTTTGTACTTGAA	Qiagen	SI00433643
siHAPIP.2	UUCAAUCCAGUCCAACACCUG	CAGGTGTTGGACTGGATTGAA	Qiagen	SI00433650
siHAPIP.3	UAGCUGACUCCGAUCUCCGUG	CACGGAGATCGGAGTCAGCTA	Qiagen	SI00433657
siIGF1R.1	UUCGUUGAGAAACUCAAUCCU	AGGATTGAGTTTCTCAACGAA	Qiagen	SI02624545
siIGF1R.2	UAUGAUGAUGCGAUUCUUCGA	TCGAAGAATCGCATCATCATA	Qiagen	SI02624552
siIGF1R.3	UAGGACUGGAUUAUUCUCCAU	ATGGAGAATAATCCAGTCCTA	Qiagen	SI00017521
siIKBKE.1	AUGAUCUCCUUGUUCGCGCGU	ACGGCGGAACAAGGAGATCAT	Qiagen	SI02622319
siIKBKE.2	UCUAAUGCUUCAGGAUGCCdTdC	GAGGCAUCCUGAAGCAUUAGA	Ambion	920
siIKBKE.3	GUACUCCAUCACCAGUACCCdTdT	AAGGUACUGGUGAUGGAGUAC	Ambion	919
siINADL.1	UUCAAGUUCAAUAAUGUGCAG	CTGCACATTATTGAACTTGAA	Qiagen	SI02635962
siINADL.2	UAUAUGGUUGAGUUGACCCUU	AAGGGTCAACTCAACCATATA	Qiagen	SI02635969
siINADL.3	UGGAUCUCUAGCAACGAGCAU	ATGCTCGTTGCTAGAGATCCA	Qiagen	SI03050313
siINPP5D.1	UUGGACACCAUGUUGAUGGGA	TCCCATCAACATGGTGTCCAA	Qiagen	SI00078582
siINPP5D.1	UUGGACACCAUGUUGAUGGGA	TCCCATCAACATGGTGTCCAA	Qiagen	SI00078582

<i>siRNA Name</i>	<i>Sequence (Guide strand)</i>	<i>Sequence (Target)</i>	<i>Supplier</i>	<i>siRNA Number/ Catalogue No.</i>
siINPP5D.2	UUGGCUGUCAACAGUCCCGGG	CCCGGGACTGTTGACAGCCAA	Qiagen	SI00078589
siINPP5D.2	UUGGCUGUCAACAGUCCCGGG	CCCGGGACTGTTGACAGCCAA	Qiagen	SI00078589
siINPP5D.3	UUGACGAACCCUAAGGAGGUU	AACCTCCTTAGGGTTCGTCAA	Qiagen	SI03029005
siINPP5D.3	UUGACGAACCCUAAGGAGGUU	AACCTCCTTAGGGTTCGTCAA	Qiagen	SI03029005
siIRAK1.1	GGAACUCUUGAUGUCUCCAdTdG	CAUGGAGACAUCAAGAGUUCC	Ambion	2242552
siIRAK1.2	UUUCCAAUUGUAAGCAGGdTdA	UACCUGCUUACAAUUGGAAA	Ambion	242551
siIRAK1.3	UACAUGAAACUGACUUGCdTdT	AAGCAAGUCAGUUUCAUGUA	Ambion	144789
siSYNA1.1	UUGGACACCUCCUAGAGCGG	CCGCTCTAAGGAGGTGTCCAA	Qiagen	SI00449015
siSYNA1.2	UUCAGCGACGAGAUGUCCCAG	CTGGGACATCTCGTCGCTGAA	Qiagen	SI00449022
siSYNA1.3	U AACUCACGAUGGACAUGGUC	GACCATGTCCATCGTGAGTTA	Qiagen	SI03101525
siKIAA1446.1	UACGGUGACAUGGUCCGCCCCG	CGGGCGGACCATGTCACCGTA	Qiagen	SI02224957
siKIAA1446.1	UAGCGUUCAGAGAAGCUGCAG	CTGCAGCTTCTCTGAACGCTA	Qiagen	SI02224964
siKIF11	N/A		Ambion	AM4639
siLMNA	UGUUCUUCUGGAAGUCCAGUU	AACTGGACTTCCAGAAGAACA	Qiagen	1022050

<i>siRNA Name</i>	<i>Sequence (Guide strand)</i>	<i>Sequence (Target)</i>	<i>Supplier</i>	<i>siRNA Number/ Catalogue No.</i>
siLOC375449.1	UUGGAAGAAGGUACCUCCCAG	CTGGGAGGTACCTTCTTCCAA	Qiagen	SI00164374
siLOC375449.2	UAAUAUGUGGUCAAGCUCCUC	GAGGAGCTTGACCACATATTA	Qiagen	SI02225482
siLOC375449.3	UAUCAAAACUCCUCUUCUGGG	CCCAGAAGAGGAAGTTTGATA	Qiagen	SI02650151
siLRPAP1.1	UUUGUGAUGCAGGAACUCCCG	CGGGAGTTCCTGCATCACAAA	Qiagen	SI00036288
siLRPAP1.2	UCGUGGAUUUCUUCGGUCCUG	CAGGACCGAAGAAATCCACGA	Qiagen	SI03069206
siLRPAP1.3	UUUCCGUCCAGACCAUACUUG	CAAGTATGGTCTGGACGGAAA	Qiagen	SI00036302
siMAD.1	UUCUUUAUGUGCAAUUUGGCU	AGCCAAATTGCACATAAAGAA	Qiagen	SI00036645
siMAD.2	UUUGUUAUAACUCAACGUA	TACGTTGAGTTTATTAACAAA	Qiagen	SI00036652
siMAPK10.1	UAUACUGGCAAUAUAUUACdAdG	CTGTAATATATTGCCAGTATA	Qiagen	SI00042651
siMAPK10.2	UUAGCUGCAAUACAGAACCdCdT	GGGTTCTGTATTGCAGCTAA	Qiagen	SI00042658
siMAX.1	AUUCUGAUUACUCCAAACCGG	CCGGTTTGGAGTAATCAGAAT	Qiagen	SI00036890
siMAX.2	AUUUCUUAGAAAUACACACGG	CCGTGTGTATTTCTAAGAAAT	Qiagen	SI00036904
siMYC.1	UUUAAGGAUAACUACCUUGGG	CCCAAGGTAGTTATCCTTAAA	Qiagen	SI00038262
siMYC.2	UGUGUAACUGCUAUAACGUU	AACGTTTATAGCAGTTACACA	Qiagen	SI02628640

<i>siRNA Name</i>	<i>Sequence (Guide strand)</i>	<i>Sequence (Target)</i>	<i>Supplier</i>	<i>siRNA Number/ Catalogue No.</i>
siNeg	N/A		Ambion	AM4611
siOR1E2.1	UUUACAAAUGACCCUUUCCAG	CTGGAAAGGGTCATTTGTAAA	Qiagen	SI00052878
siOR1E2.2	UUGGUUCUGCAUGUUCUGCAG	CTGCAGAACATGCAGAACCAA	Qiagen	SI00052885
siOR1E2.3	UUGGGCAUUGUGACUGAGGAA	TTCCTCAGTCACAATGCCCAA	Qiagen	SI02631272
siPDE11A.1	UUGAUCGAAAUUAUUCACGAU	ATCGTGATATATTTGATCAA	Qiagen	SI00117936
siPDE11A.2	UUGUGGUUAUAGAACCAUCCGA	TCGGATGGTTCTATACCACAA	Qiagen	SI00117922
siPDE11A.3	UUACACGUGAACCAAUGUUU	AAACATTTGGTTCACGTGTAA	Qiagen	SI00117943
siPPP2CB.1	UAUACUUGGGUAAUUUGUCGG	CCGACAAATTACCCAAGTATA	Qiagen	SI02225797
siPPP2CB.2	UUA AUGCCAAGACAGAUCCCA	TGGGATCTGTCTTGGCATTAA	Qiagen	SI02225804
siPRKAA2.1	GACCUGCAUACAAUCUGCCdTdG	CAGGCAGAUUGUAUGCAGGUC	Ambion	130652
siPRKAA2.2	UGUUGGAGUGCUGAUCACCCdTdG	CAGGUGAUCAGCACUCCAACA	Ambion	103599
siPRKAA2.3	AUAAUGUCAUACGGUUUGCdTdC	GAGCAAACCGUAUGACAUUUAU	Ambion	142926
siPRKAB2.1	UUUAGGUGCAUCAAUGAGUU	AACTCATTTGATGCACCTAAA	Qiagen	SI00076531
siPRKAB2.2	UAAAUGAGUAGUACUUUGGGU	ACCCAAAGTACTACTCATTTA	Qiagen	SI00287777

<i>siRNA Name</i>	<i>Sequence (Guide strand)</i>	<i>Sequence (Target)</i>	<i>Supplier</i>	<i>siRNA Number/ Catalogue No.</i>
siPRKAB2.3	UAAUGGACAAUGCAUAGAGAU	ATCTCTATGCATTGTCCATTA	Qiagen	SI03048822
siPRKCD.1	AUUGCCUGCAUUUGUAGCCdTdT	AAGGCUACAAAUGCAGGCAAU	Ambion	
siPRKCD.2	GCUCAUGUAGUUGUGAACCdTdT	AAGGUUCACAACUACAUGAGC	Ambion	130653
siPRKCD.3	GUCUUCACACUUUAUCCcdTdT	CAGGGAUAAAGUGUGAAGAC	Ambion	103702
siPRKCQ.1	AGGUU AACAGCCUCGCCcdTdT	CAGGGCGAGGCUGUUAACCU	Ambion	784
siPRKCQ.2	GAUCUGCAUGACUCUUCcdTdT	AAGGGAAGAGUCAUGCAGAUC	Ambion	782
siPRKCQ.3	UUUGUGCAAGAUAAAUCcdTdT	GAGGAUUUAUCUUGCACAAA	Ambion	783
siPRKRIR.1	AAUUUGAGUUGUCCCAUGACUGAGG	CCTCAGTCATGGGACA ACTCAAATT	Invitrogen	HSS108577
siPRKRIR.2	UUCACCAGAAUUUAUCCGACACUCC	GGAGTGTCGGATAAATTCTGGTGAA	Invitrogen	HS108578
siPRKRIR.3	AUGAGAUUCAUCAACAACCUCACC	GGTGAGGTTGTTGATGAATCTCAT	Invitrogen	
siPTP4A3.1	UAUUGUGCAAUACUUGGGUG	CACCCAAGTATTTGCACAATA	Qiagen	SI00094430
siPTP4A3.2	UUCGGGUGUCCGGAGCACCUG	CAGGTGCTCCGGACACCCGAA	Qiagen	SI03072202
siPTP4A3.3	UGC UUGUCCAAGAGAAACGAG	CTCGTTTCTCTTGGACAAGCA	Qiagen	SI03092285
siPTPRV.1	UAGAUAUACUGCUUCAGCGUU	AACGCTGAAGCAGTATATCTA	Qiagen	SI00695863

<i>siRNA Name</i>	<i>Sequence (Guide strand)</i>	<i>Sequence (Target)</i>	<i>Supplier</i>	<i>siRNA Number/ Catalogue No.</i>
siPTPRV.2	UAUAGGUCCACUCUGUGGCAU	ATGCCACAGAGTGGACCTATA	Qiagen	SI00695884
siPTPRV.3	UCAGAUAGCAAGCGCUCCCUG	CAGGGAGCGCTTGCTATCTGA	Qiagen	SI02812502
siRAD9A.1	UAGAAUUCCAAGUCAGCGCCA	TGGCGCTGACTTGGAATTCTA	Qiagen	SI02633617
siRAD9A.2	UAGCUGCGCAGGAUGACCCUG	CAGGGTCATCCTGCGCAGCTA	Qiagen	SI00065618
siRAD9A.3	UUGAUGGUGAAGAUGGCGGGC	GCCCGCCATCTTCACCATCAA	Qiagen	SI00065625
siROS1.1	UAAGUAAGAAGGUCUCCUCUU	AAGAGGAGACCTTCTTACTTA	Dharmacon	D-003173-05
siROS1.2	UAAUCCUGAACCUCUGUAAUU	AATTACAGAGGTTTCAGGATTA	Dharmacon	D-003173-06
siROS1.3	UUCAUGCUUAGGUUUGUUCUU	AAGAACAACCTAAGCATGAA	Dharmacon	D-003173-07
siROS1.4	UUAUUUGAAGUGCUCUUUCUU	AAGAAAGAGCACTTCAAATAA	Dharmacon	D-003173-08
siSHARPIN.1	UUCAAGUGUGACCUUGCAGCCU	AGGCTGCAGGTCACACTTGAA	Qiagen	SI00140189
siSHARPIN.1	UUCAAGUGUGACCUUGCAGCCU	AGGCTGCAGGTCACACTTGAA	Qiagen	SI00140189
siSHARPIN.1	UCAAGUGUGACCUUGCAGCCUG	CAGGCTGCAGGTCACACTTGA	Qiagen	SI00140175
siSHARPIN.2	UCAAGUGUGACCUUGCAGCCUG	CAGGCTGCAGGTCACACTTGA	Qiagen	SI00140175
siSMAC.1	UUCAGUAAUAGCUUCAAUUCdAdA	TTGATTGAAGCTATTACTGAA	Qiagen	SI00124621

<i>siRNA Name</i>	<i>Sequence (Guide strand)</i>	<i>Sequence (Target)</i>	<i>Supplier</i>	<i>siRNA Number/ Catalogue No.</i>
siSMAC.2	UUUCUGUGCAAUAGGAACCDGdC	GCGGTTCCCTATTGCACAGAAA	Qiagen	SI00124628
siSRP72.1	UUCAGAAGGAAUAAAUACCDtDg	CGGTATTTATTCCTTCTGAA	Qiagen	SI00733327
siSRP72.2	UUAUUGGCUAACACUUUGGdTDg	CCCAAAGTGTTAGCCAATAA	Qiagen	SI00733334
siSTK32B.1	UAUUGUAUAAAUAGGGAGCCG	CGGCTCCCTATTTATAACAATA	Qiagen	SI00121597
siSTK32B.2	AUACCGAUUGGAUACUCUCUG	CAGAGAGTATCCAATCGGTAT	Qiagen	SI00121618
siSTK32B.3	UUCUUGAACACCGCGUCCCAG	CTGGGACGCGGTGTTCAAGAA	Qiagen	SI02224859
siTAOK1.1	UCGCUUAUUUAUUUCCAGCUG	CAGCTGGAATATAATAAGCGA	Qiagen	SI00739410
siTAOK1.2	UUCAGUAGUACUUUAGCACUG	CAGTGCTAAAGTACTACTGAA	Qiagen	SI00739417
siTAOK1.3	UUGCGGACUCCCAUACUGCUA	TAGCAGTATGGGAGTCCGCAA	Qiagen	SI03110968
siTEGT.1	UUGAUCUCCAUGUUCGGCCUU	AAGGCCGAACATGGAGATCAA	Qiagen	SI02781254
siTEGT.2	UACCUUGAUACACAUAUUCAG	CTGATTATGTGTATCAAGGTA	Qiagen	SI02781282
siTLR4.1	UAACUUUGGAAUGACAUCGAG	CTCGATGTCATTCCAAAGTTA	Qiagen	SI02630789
siTLR4.2	UUAGUAGGCAAUAACUUUGGA	TCCAAAGTTATTGCCTACTAA	Qiagen	SI00151011
siTLR4.3	UUAUCAGCCCAUAUGUUUCUG	CAGAAACATATGGGCTGATAA	Qiagen	SI02630796

APPENDIX B SEQUENCE OF OLIGONUCLEOTIDE PRIMERS USED FOR QUANTITATIVE PCR

<i>siRNA Name</i>	<i>Sequence (Guide strand)</i>	<i>Sequence (Target)</i>	<i>Supplier</i>	<i>siRNA Number/ Catalogue No.</i>
siTNFRSF10A	AUAUAUUAUGUCCAUUGCCdTdG	CAGGCAAUGGACAUAUAUAU	Ambion	5004
siVPS16.1	CUUCGUUUUUGUUUUGUUGUU	AACAACATAACAAATACGAAG	Dharmacon	D-013003-01
siVPS16.2	UUGGCUGCAAACUCAUUCUUU	AAAGAATGAGTTTGCAGCCAA	Dharmacon	D-013003-02
siVPS16.3	UAGCCAAUGGGUGAUUUCUUU	AAAGAAATCACCCATTGGCTA	Dharmacon	D-013003-03
siVPS16.4	CUGCUCUGCACGCUUGUUGUU	AACAACAAGCGTGCAGAGCAG	Dharmacon	D-013003-04
VPS16 smartpool	Pool		Dharmacon	M-013003-00- 0005

Appendix B Sequence of oligonucleotide primers used for quantitative PCR

Primers were designed and tested as outlined in 2.4.1 and 2.3.7. Primers with ‘-’ genomic product length either cross exon boundaries or the genomic product would be longer than 20kb. Primers with no efficiency listed generated no product and so efficiency could not be determined.

<i>Gene</i>	<i>Forward</i>	<i>Reverse</i>	<i>Product Length (Genomic)</i>	<i>Product Length (cDNA)</i>	<i>Efficiency</i>
ABL2	TGGATTACCTCCGAGAATGC	CCACATGGTTTTCTCCCACT	1229	159	1.995
ACO1	AGGTCAAGCTGGATACTGGCA	ACATCAGTGTCAAACCTCATGACA	0	58	2.038
ACTB ¹	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	0	140	1.950
ADORA1	AGCCTCCTCTCCCTCTGTG	CTGCCTGACTGTTCTGTCCA	37202	126	-
AIFM3	GCAAAGGCAAAGAAGTGGAG	CACCACAGACACAGAGTGGG	279	167	1.997
ARSA	ACCTGAGACCATGCGTATGT	GGTCAAAGCCATCCAAGGTG	314	224	2.141
BID	CCGCTTGGGAAGAATAGAGG	CCAGCATGGTCTTCTCCTTC	5681	248	1.861
CASP3	TGTTTGTGTGCTTCTGAGCC	CGGCCTCCACTGGTATTTTA	840	233	2.022
Casp8	TCCAAATGCAAACCTGGATGA	GGGCACAGACTCTTTTCAGG	1089	73	2.078
CCNT1	GCCAATCTGCTTCAGGACA	TGCTAGAAACAAGGCTGCTG	10686	150	1.947

<i>Gene</i>	<i>Forward</i>	<i>Reverse</i>	<i>Product Length (Genomic)</i>	<i>Product Length (cDNA)</i>	<i>Efficiency</i>
DCK	AAAGCCTTGAATTGGATGG	CGAAGTTGGTTTTTCAGTGTC	0	174	-
DIABLO	AGCTGGAAACCACTTGGATG	CAGCTTGGTTTCTGCTTTCC	8080	170	1.866
DYRK1A	TCCTTGATAGGCAAAGGTTCC	AACCCATTCTTGCTCCACAC	5709	69	1.934
ETNK1	GATCCAAAGCATGTCTGCAA	TGTGTGCATGAATAGCATGG	15073	82	1.983
FBXO11	CAAAATCTGGGGAGGACAGA	ACACCTGCTTGAGCATTCT	3457	237	2.045
FLJ32312	TTCAGTCTCCTTCCCACCAC	CTCTGCTTTCCATTTCCTG	0	81	1.920
GAPDH ¹	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	0	87	2.005
GPR132	GAATGTGAATGTGCCCAATG	ACACGTTGTTGCAGGTCTTG	3401	117	-
GUK1	CTTCAGCGTGTCCCATAACC	GCATCACCTCCCTGGTTACA		86	2.042
HAPIP	GCGCATCTTTGAGCAGTACA	CTTCATTCCAGGCGTCTAGC	14657	59	-
IGF1R	AAAAACCTTCGCCTCATCCT	TGCAAGTTCTGGTTGTCGAG	9143	80	2.156
IKBKE	TCCCTCCTCTACCTCAGCAG	GGATCTCAGGCGTTCCAG	4527	70	1.882
INADL	GGCGTGGTTGTGAGGACTAT	AAGATGTGGTCCCCTGTCTG	12496	77	1.993
INPP5D	GACACAGAAAGTGTCGTGTCTC	GGAACCTCCTTGGCCTCACAG	6845	57	-

<i>Gene</i>	<i>Forward</i>	<i>Reverse</i>	<i>Product Length (Genomic)</i>	<i>Product Length (cDNA)</i>	<i>Efficiency</i>
IRAK1	GGTTTCGTCACCCAAACATT	GGGGCTGTCCTGATGTAGAA	1147	209	1.941
ISYNA1	CGCAAGGAGGCCAACTACTA	GTCCCAGCCATCGAACAC	0	147	1.767
KIAA1446	CGCAGACATGGAGAAACTCA	TCGAGCTTGTGTGTGGTGTAG	21312	84	1.940
LIMK2	ACCCAATGTGCTCAAGTTC	GCCCTTTTCCTCTCTTCCAC	4794	295	1.734
LMNA	GGCGAGGAGGAGAGGCTA	CCTGTGTCTGGGATGAGTGA	0	88	1.891
LOC375449	GTCTCCCCAGAAGAGGAAGTT	GGTGATGTTCTCCCAGCATT	0	55	1.958
LRPAP1	GGGGAGAAGGAAGCGAGA	TCCGTCCAGACCATACTTGG	4779	66	1.882
MAD	ACATGGTTATGCCTCCATGTTAC	AGATGAGCCCGTCTATTCTTCTC	0	135	2.046
MAPK10	AACTCAAAGCCAGCCAAGC	GTAAGGCGTCGTCCACTGAT	0	87	1.824
MAX	CAGTCCCATCACTCCAAGG	TGTGGTTTTTCCTTCGCATA	15764	93	2.076
MTMR3	AGCAGAGTGGGCTCAGTGTT	ACTGTCCACGTTTGGTCCTC	230	230	1.968
MYC	CAGCTGCTTAGACGCTGGATT	GTAGAAATACGGCTGCACCGA	1744	131	1.991
OR1E2	ACTACACCGCCATCTGCTTC	ACCACGGAGAGACAGAGCAT	70	70	1.747
PDE11A	ATGCCTTCAACGTGTGTCAG	CCCACAATCACCGCTAAAAT	15876	97	-

<i>Gene</i>	<i>Forward</i>	<i>Reverse</i>	<i>Product Length (Genomic)</i>	<i>Product Length (cDNA)</i>	<i>Efficiency</i>
PPP2CB	GCTGAACGAGAACCAAGTGC	CAGTAACAGGGCAACGAACC	0	95	1.938
PRKAA2	ACCCACTGAAACGAGCAACT	ATAAGCCACTGCAAGCTGGT	0	218	1.942
PRKAB2	GTGTTTCAGCCTCCCTGACTC	TCCTGCTGCCATGATACAAA	4117	62	2.027
PRKCD	GCCTCAACAAGCAAGGCTAC	AGGTGGGGCTCATGTAGTTG	1819	186	1.879
PRKCQ	GAATCAGAGAACGGGCAGAT	TGGGCATCAAAAGTGCTGT	0	77	1.866
PRKRIR	CTTGGCCTTCTTCAGGTTCC	AGTTCTCCACCCACTTCTGG	0	59	-
PTP4A3	AGAGCGGGATGAAGTACGAG	TTTCTCCAGGTAGGTGAGCTG	3194	93	1.837
PTPRV	CCTAGAATCCCAGACATTGGCA	GCTGGTTGTTGCTTGGAGGTT	4654	65	-
RAD9A	GTGAAGGTGCTCGGCAAG	AGAAAGCAGGCATAGGCAGA	0	128	1.895
RBX1	GTGGAATGCAGTAGCCCTCT	GTTTTGAGCCAGCGAGAGAT	14286	192	1.997
ROS1	TTCATTCACAGGTCTTTTGGGA	GCTGGATAAGGCTGATGACC		68	2.086
Sharpin	GGACAGAATGGTCACAAGAGC	GACTGGGCAGGGAGACAG	0	152	1.813
SRP72	CTGAACCAGGCCATGAAAAT	CAAAGCCTCCTCTGTTTCGAC	4120	165	1.972
STK32B	ACATCTGTGAGCTGGCACTG	GCAGGATATTGTCTGGCTTGA	0	84	1.972

<i>Gene</i>	<i>Forward</i>	<i>Reverse</i>	<i>Product Length (Genomic)</i>	<i>Product Length (cDNA)</i>	<i>Efficiency</i>
TAOK1	CGAGCTTGAACAGAGGGTCT	CTCATTCTGCAAAGCCAACA	8342	82	2.017
TEGT ²	ACGGACTCTGGAACCATGAA	AGCCGCCACAAACATACAA	564	141	1.984
TLR4 ³	ACAACCTCCCCTTCTCAACC	TGAGATGTCCAATGGGGAAG	3759	300	-
TNFSRF10A	AGAGAGAAGTCCCTGCACCA	GTCACTCCAGGGCGTACAAT	1201	154	1.865
VPS16	CTTCTTGTTGGCGATGTGG	GCCCGTTGAATCTTGTCAG	0	131	1.891

¹ Oligo sequence from RTPrimerDB {{260 Pattyn,Filip 2006; }}

² Oligo sequence from {{493 Grzmil,Michal 2003; }}