Analysis of Genes Controlling Notochord Development in Zebrafish

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Abstract

The notochord is a vital and defining organ in vertebrates. Mutagenesis screening in zebrafish identified seven 'dwarf' mutants that lack notochord development; *grumpy, sleepy, bashful, dopey, happy, sneezy* and *doc*. This thesis is concerned with the identification and positional cloning of the *doc* locus as well as the confirmation and characterisation of the *dopey* and *happy* loci. Previous positional cloning efforts identified the *grumpy, sleepy* and *bashful* genes, demonstrating a requirement for the laminin chains β1, γ1 and α1 in formation of the notochord basement membrane (Parsons et al., 2002b; Pollard, 2002) and the mutant *sneezy*, which has been shown to encode the COPI subunit α (Coutinho et al., 2004).

This thesis establishes that the *doc locus* lies within a 0.5Mb region on linkage group 18, containing several genes, including a novel gene encoding a predicted protein with 14 WD40 domains. Antisense morpholino (MO) knock-down of *doc* results in a phenocopy of *doc* and *insitu* expression demonstrates that this gene is expressed specifically within the notochord during development. Expression analysis of *echidna hedgehog* (*ehh*) demonstrated that MO knock-down of this gene results in a lack of notochord differentiation. I therefore expect this novel gene is *doc*.

Analysis of the mutants *dopey* and *happy* has demonstrated that they encode the coatomer subunits COPβ' and COPβ respectively. Expression of these and other COPI subunits demonstrate that the majority of COPI subunits are up-regulated within the notochord during development and maintained abnormally in COPI deficient embryos. I have investigated the mechanism of coatomer gene regulation

and found that loss of coatomer function leads both to up-regulation of coatomer mRNA and activation of the unfolded protein response (UPR). Suggesting that the UPR is the regulator of mRNA expression, functioning to maintain the secretory network during development, though work to provide definitive proof remains.

To my mum, who taught me and to Zoë, who keeps me sane.

It is not enough to have a good mind; the main thing is to use it well.

Rene Descartes 1596-1650

Creationists make it sound as though a “theory” is something you dreamt up after being drunk all night.

Isaac Asimov 1920-1992

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Abbreviations

AP	Antero-posterior
BAC	Bacterial artificial chromosome
bal	*bashful*
BCIP	X-phosphate/5-Bromo-4-chloro-3-indolyl-phosphate
BM	Basement membrane
BSA	Bovine serum albumin
COPI	Coatomer complex
DEPC	Diethylpyrocarbonate
DIG	Digoxygenin
dop	*dopey*
DTT	Dithiothritol
DV	Dorso-ventral
EDTA	Ethylene-diamine-tetra-acetate
EST	Expressed sequence tag
GTP	Guanidine 5'-triphosphate
gup	*grumpy*
hap	*happy*
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hpf	Hours post-fertilisation

IPTG	Isopropylthio-β-D-galactosidase
LG	Linkage group
MO	Antisense morpholino oligonucleotide
NBT	4-Nitro blue tetrazolium chloride
OD	Optical density
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RAPD	Random amplified polymorphic DNA
RH	Radiation hybrid
SDS	Sodium dodecyl sulphate
sly	*sleepy*
sny	*sneezy*
SSLP	Simple sequence length polymorphism
TAE	Tris, acetate, EDTA
TBE	Tris, borate, EDTA
TE	Tris EDTA
UPR	Unfolded Protein Response
WT	Wild Type
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
YSL	Yolk Syncytial layer

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It hasn't always been simple, but it's been fun, mostly, and I will remember these years with great fondness. Thank you all.

Chapter One

Introduction

1 Introduction

In this section I shall provide a brief review of the zebrafish as a laboratory model organism, highlighting the features that make the zebrafish a highly advantageous system to study vertebrate development. I will also provide an overview of early zebrafish embryology and examine in some detail the molecular mechanisms and processes involved during development. I will pay particular attention to the development of the organiser and its derivative, the notochord. I will discuss knowledge derived from work on several model organisms, relating this to the development of the zebrafish. I will review the recent advances made in understanding processes involved in notochord differentiation, much of which stems from work on mutants generated as part of ENU mutagenesis screens (Development **123**, 1996). I will also discuss the structure and function of the notochord, emphasising its importance throughout development.

1.1 Overview of *Danio rerio*

1.1.1 The zebrafish as a model organism

The zebrafish (*Danio rerio*) has, in recent years, become a widely accepted model organism in the study of vertebrate developmental biology. The zebrafish exhibits many features that have helped make it a choice system for studying the processes of developmental biology. The zebrafish is a cheap and easy organism to

maintain, has a relatively short breeding cycle, taking only three months until it begins reproducing, a high fecundity, and produces relatively large (~0.7mm) translucent embryos that can be obtained throughout the year. The optical clarity of the embryo allows direct visualisation of individual cells and the cell movements that occur within the developing embryo. This visual accessibility, coupled with the short life cycle (a zebrafish embryo is fully formed and patterned by 5 days post fertilisation) and the external fertilisation of the zebrafish egg, makes studying the developmental processes of the zebrafish a relatively easy task. The short life cycle and high fecundity also make genetic studies a much easier venture.

As such, the zebrafish seemed the ideal organism on which to carry out the first vertebrate mutagenesis screen, similar to those carried out on *Drosophila* in the early 1980s (Nusslein-Volhard and Wieschaus, 1980). In 1996 the results of such a screen were published and an entire issue of Development (**123**; 1996) was dedicated to the characterisation and description of several hundred of the thousands of mutants isolated (Driever et al., 1996; Haffter et al., 1996). Thus, the zebrafish has a highly desirable and advantageous resource, a vast number of mutants with specific developmental defects. These mutants have been a source of intense study, helping to promote the zebrafish as a model of vertebrate development. The characterisation of these mutants and the molecular processes affected therein continue to reveal fascinating insights into the pathways involved during vertebrate development (Currie and Ingham, 1996; Holder and McMahon, 1996; Roush, 1996).

1.1.2 Brief summary of zebrafish embryology

In the first 72 hours following fertilisation, a zebrafish embryo develops from a single cell to free-swimming larvae with all its major axes and structures patterned. This 72 hour period can be separated into seven distinct phases: zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching.

The zygote stage extends from fertilisation until the time of first cleavage and covers the first 40 minutes following fertilisation at 28ºC. During this time, the yolk cell, which consists of both yolk and cytoplasm, undergoes cytoplasmic streaming, where the cytoplasm separates from the yolk and segregates in the animal pole to form the blastodisc. This segregation continues beyond the zygote phase and into cleavage phase. After the first cleavage, the cells, known as blastomeres, undergo synchronous meroblastic cleavages every 15 minutes (Kimmel and Law, 1985). These six cleavages are confined to the animal pole and occur at regular orientations, resulting in a predictable pattern of blastomeres that is dependant on the number of cleavages that have occurred. The sixth cleavage is the first to occur in the horizontal plane, and results in a two-tiered arrangement of cells. This regular succession of cleavages continues until the tenth division, which correlates with the start of the mid-blastula transition (MBT) (Kane and Kimmel, 1993).

The MBT, which marks the beginning of zygotic transcription, occurs during the blastula period, which begins at the 128-cell stage (the 8^{th} cleavage), and continues until the start of gastrulation. During the early stages of the blastula period, cell divisions occur with some degree of synchrony, so that divisions can be seen as a wave that originates at the animal pole and then spans out to the marginal cells. It is only once the MBT occurs, at the 512-cell stage (the 10^{th} cleavage), that all synchronicity of division is lost (Kane and Kimmel, 1993). At cycle ten, cells can be divided into three distinct layers; the enveloping layer (EVL), which forms the

periderm, a layer that acts to surround and protect the developing embryo; the deep cell layer, which develops into the embryo proper and the yolk syncytial layer (YSL), which is thought to drive epiboly and to pattern the embryo at early stages. The YSL is formed when blastomeres of the marginal tier, which have remained cytoplasmically linked to the yolk cell, collapse, causing the release of their cytoplasm and nuclei into the adjoining yolk cells cytoplasm, generating the YSL. The nuclei then continue to undergo division after YSL formation, though after 3 divisions this ceases, with nuclei becoming enlarged, possibly indicating transcriptional activation (Kane et al., 1992), (Trinkaus, 1992).

As the embryo continues to develop, it eventually undergoes epiboly, which is the first major cell/morphogenetic movement of the developing embryo. Epiboly involves a coordinated movement of the cells of the blastoderm from their animal location towards the vegetal pole, so as to surround the yolk cell. The force necessary for this movement is generated through connections between the marginal cells of the EVL and the YSL, which is itself attached to force generating microtubules within the yolk cell. The gastrulation movements of involution, which marks the beginning of gastrulation at 50% epiboly when cells of the germ ring are subducted to form multiple layers, and of convergent extension, where cells stream to the dorsal side of the developing embryos, occur alongside epiboly movements (Solnica-Krezel et al., 1995). It is during epiboly that the hypoblast, a layer of cells residing between the epiblast and the yolk cell, is specified at the germ ring, the major axes of the embryo are also established and cells are first specified to distinct fates.

At approximately 50% epiboly, involution of marginal cells begins, forming the germ ring, which is visible as a thickening of the marginal region. This marks

the beginning of gastrulation, which acts to produce the three layered body plan of triploblastic organisms. There is a pause in epiboly shortly after 50% (approximately 20 minutes at 24ºC) at which point the embryo reaches shield stage, so named due to the formation of the dorsal organiser, called the embryonic shield in zebrafish. The shield marks the first obvious morphological identifier of the dorsal side and is the zebrafish equivalent to the node in mouse, Hensen's node in chick and Spemann's organiser in amphibians. Studies in zebrafish have established that transplantation of the shield to the ventral side of a host embryo is able to induce the formation of a complete secondary axis (Saude et al., 2000; Shih and Fraser, 1996). As epiboly continues, cells begin to converge on the dorsal region, the embryo extends along the Anterior-Posterior (AP) axis and the shield differentiates to form the axial mesoderm, which includes the notochord.

By approximately 10 hours post fertilisation, the embryo has reached tailbud stage. This stage marks the end of gastrulation, all major body axes are formed and the three germ layers are specified and organised. As the embryo progresses beyond tailbud, it begins the segmentation period, as first defined by formation of the somites. It is during during this stage that the embryo begins to elongate and tissues begin to differentiate. One of the earliest tissues to fully differentiate is the notochord.

Somitogenesis begins at the start of the segmentation period and represents one of this periods major events. Around 30 to 34 somites are formed, sequentially, in blocks along the AP axis from paraxial mesoderm. Somites form in pairs on either side of the notochord in the trunk and tail. The somites are blocks of undifferentiated mesenchyme surrounded by an epithelial layer and eventually differentiate into myotome and sclerotome, which will differentiate into segments of body muscle and

vertebral cartilage, respectively. The notochord plays a vital role in the choice between these two fates. The somites represent one of three segmental structures that form during this period; the other structures being rhombomeres, within the CNS, and the pharyngeal arches, which form the jaw and gills. The nervous system develops concurrently with somitogenesis, during which the neural plate undergoes an obvious thickening along the embryonic axis and the polster, a structure that will later form the hatching gland, develops at the anterior end. Analysis of neural markers makes it evident that, even at this early stage, a large degree of patterning has already taken place. By 24 hpf, the segmentation period is coming to an end, as characterised by a completion of somite formation, differentiation of blood and the first heartbeats. By this stage, the first fully differentiated structure, the notochord, has formed.

The final period before hatching occurs is known as the pharyngula period. Several structures necessary for the development into a free swimming and feeding larva are elaborated during this period, including, most obviously, the fins, jaws and gills. After two days, hatching of the developing embryo occurs. By approximately 4 days, all major organ systems have completed their extensive morphological movements. Hence, just 96 hours after fertilisation, the embryo has developed into a complex free-swimming fish. The events that occur during this time characterise the major challenges of developmental biology. The major aim of this thesis is to enhance the current understanding of the processes involved in the development of the notochord.

1.2 Early zebrafish Development

Recent work in the field of developmental biology has vastly increased our understanding of the stages and processes that occur during early vertebrate development. Advances in the field of molecular biology have made forward genetic studies increasingly plausible and the combination of these with classical embryological work and reverse genetic screens have revolutionised our understanding of the molecular and cellular processes involved during development. In the following sections the current understanding of the molecular processes involved in early development, specifically, the processes that are involved from fertilisation until gastrulation, will be discussed, including the mechanisms involved in the establishment of the three germ layers (ectoderm, endoderm and mesoderm), in forming the major body axes and in early patterning. It is during these early stages that the organiser is formed. The organiser constitutes a vital signalling centre that will eventually form the prechordal plate, hatching gland and, most relevantly to this thesis, the chordamesoderm, which itself differentiates to form the notochord.

Much of what is known about dorsal specification has been established through work on the systems involved in amphibian development. Such work has demonstrated that these amphibian systems are highly similar to those involved in the development of other vertebrate embryos. Thus, much of what is discussed concerning organiser will describe knowledge of *Xenopus* development, with comparisons and parallels being made to what has been established in the zebrafish.

1.2.1 Formation of the dorsal Organiser

Experiments published in 1924 by Spemann and Mangold identified the dorsal organiser, by virtue of its ability to induce a secondary axis when transplanted to the ventral side of a host embryo. The organiser functions through intercellular signals that act in several distinguishable roles. The organiser acts to dorsally pattern to the mesoderm, induces convergent-extension movements of the ectoderm and mesoderm and induces neurectoderm, providing signals to pattern the neurectoderm along the anterior-posterior axis (Harland and Gerhart, 1997). Transplantation studies have shown that structures equivalent to the amphibian Spemann organiser are present in the embryos representing the major vertebrate phyla. In teleost fish, such as zebrafish, the dorsal organiser is known as the embryonic shield (Saude et al., 2000; Shih and Fraser, 1996). In avians, the dorsal organiser is known as Hensen's node, and in mammals, the node (Beddington, 1994; Waddington, 1932). To easily understand the formation and specification of the organiser, it is both convenient and easy to divide organiser development into two processes; the determination of the dorsal side and the induction of mesoderm.

Dorsal specification in amphibians relies on the translocation of maternal, vegetally localised factors to the future dorsal side. By the first cleavage of an amphibian zygote, the vegetally localised dorsalising factors are segregated by a process known as cortical rotation (Gerhart et al., 1989; Vincent and Gerhart, 1987). However, recent work has suggested that cortical rotation may not be the only method of segregation. Initially, the observation that the vegetally localised dorsalising activity is broadly distributed during cortical rotation (Kageura, 1997; Sakai et al., 1996) and the observation that membrane bound organelles were able to

translocate faster than movement solely by cortical rotation would allow (Rowning et al., 1997). Further work, coupling dorsalising agents with GFP again demonstrated the rapid dorsally directed movement of the particles and demonstrated a link between the organisation of microtubules and the movement of the dorsalising factors (Miller et al., 1999; Weaver et al., 2003).

However the translocation of vegetally localised dorsal determinants occurs, the event itself acts to establish a group of vegetal cells, shown by Nieuwkoop to be capable of inducing a full secondary axis without itself contributing to the axial tissues and named, in honour of this fact, as the Nieuwkoop centre (Nieuwkoop, 1973). This group of cells then act as a signalling centre that induces the formation of organiser. However, neither the dorsal determinants, nor the Nieuwkoop centre signals are understood in precise detail. A clue to their identity was initially provided by the observation that the secreted signalling molecule, Wnt1, could induce a secondary axis in *X. laevis* when over expressed (McMahon and Moon, 1989). The details of Wingless/Wnt signalling in *Drosophila melanogaster*, in particular the protein armadillo, which was shown to play a key role in Wingless signal transduction, helped resolve the factors responsible for Wnt1's dorsalising ability (Peifer et al., 1991; Peifer and Wieschaus, 1990; Riggleman et al., 1989). The vertebrate homologue of armadillo, β-catenin, was known to be associated with the cell adhesion complexes of the Cadherin class and antibodies directed against β-catenin were found to result in axis duplication in *Xenopus* (McCrea et al., 1993). Over expression of β-catenin in either *X. laevis* or zebrafish was found to induce formation of a full secondary axis (Funayama et al., 1995; Kelly et al., 1995). Although the precise method of β-catenin's action in inducing organiser formation is not understood, it is clear that it is not β-catenin itself, as the vegetal cytoplasm of β-

catenin depleted embryos is still able to induce a secondary axis in host embryos (Marikawa and Elinson, 1999). However, recent evidence has provided likely candidates in the form of upstream factors involved in the stabilisation of β-catenin, such as GBP, *dishevelled* and GSK3 binding protein, which have been shown to be essential for dorsal specification (Miller et al., 1999; Weaver et al., 2003; Yost et al., 1998). In concert with transcription factors of the TCF/LEF family, β-catenin induces the expression of genes such as *siamois* and *twin* in *X. laevis,* which are thought to participate in organiser specification (Laurent et al., 1997; Lemaire et al., 1995; Moon and Kimelman, 1998; Nelson and Gumbiner, 1998).

Thus, cortical rotation in amphibia, which has been shown to be microtubule dependent, leads to the stabilisation and activation of β-catenin and the subsequent formation of a Nieuwkoop centre. The equivalent process in teleost fish is not clear, but does apparently culminate in the localised activation of β-catenin at the dorsal side. Direct manipulations of developing zebrafish embryos have been particularly helpful in defining the zebrafish equivalent of a Nieuwkoop centre. For example, in studies where the vegetal third of the yolk cell is removed within 20 minutes post-fertilisation, the embryo becomes completely ventralised (Ober and Schulte-Merker, 1999). Such embryos lack all dorsal mesoderm, neurectoderm and the most anterior 14-15 somites, indicating that a dorsal determinant, localised vegetally within the yolk, acts to specify the organiser. In other studies, disruption of microtubules within the early embryo has shown that an activity, located in the vegetal hemisphere and dependant on microtubule transport, is necessary for the formation of the shield and the construction of correct axes in the embryo (Jesuthasan and Stahle, 1997). Thus, although no obvious cortical rotation takes place in activated zebrafish eggs, a microtubule dependent process, possibly

analogous to that in *Xenopus*, is apparently required for the proper activation of β-catenin in the correct region.

The maternal mutant *ichabod* provides additional clues as to the nature of β-catenin localisation and activation. Mutant embryos are severely ventralised and closely resemble the ventralisation generated via removal of the vegetal yolk region. Embryos from a homozygous mutant mother can be rescued through injection of β-catenin (Kelly et al., 2000). Thus, it can be suggested that activation of β-catenin, specifically on the dorsal side by some unknown factor, possibly involving *ichabod,* produces organiser inducing activity, which may reside in the YSL, marginal blastomeres or both (Schneider et al., 1996). Indeed, recent work has provided evidence to suggest that the zebrafish functional equivalent of the Nieuwkoop centre is distributed between both the YSL and the dorsal marginal blastomeres. Injection of RNAse has shown that RNAs within the YSL are required for its ventrolateral and mesodermal inductive capabilities, as well as the proper expression of Nodal related genes in ventrolateral marginal blastomeres (Chen and Kimelman, 2000). However, it was also shown that YSL localised mRNAs are not essential for the induction of the dorsal mesoderm, suggesting that dorsal specification is due to the stabilisation of β-catenin in dorsal marginal blastomeres.

Dorsal activation of β-catenin in *X. laevis* is known to induce the expression of organiser specific homeodomain transcription factors, including *siamois* and *twin* (Laurent et al., 1997; Lemaire et al., 1995; Moon and Kimelman, 1998; Nelson and Gumbiner, 1998). The zebrafish gene *bozozok*/*dharma*/*niewkoid (boz)* encodes a homeodomain containing protein that is also regulated by β-catenin (Koos and Ho, 1998; Shimizu et al., 2000; Yamanaka et al., 1998). *boz* mutant embryos show a

complete lack of the axial mesendoderm tissues of notochord and prechordal plate (Fekany et al., 1999; Koos and Ho, 1999). Thus, though *boz* differs from *siamois* and *twin* in primary sequence, they appear to have similar roles in organiser specification and there are several lines of evidence that suggest a role for *boz* downstream of β-catenin in organiser specification (Ryu et al., 2001). Over expression of a mRNA encoding a constitutively active β-catenin is able to induce *boz* expression in wild type embryos and is also able to induce axis duplication in *boz* mutant embryos. However, this does not rescue the lack of axial mesendoderm phenotype. Contrasting this, over expression of a constitutively active type I activin receptor, Taram-A, in *boz* mutant embryos is able to induce both axis duplication and rescue the loss of axial mesendoderm (Fekany et al., 1999; Renucci et al., 1996). Finally, injection of *boz* mRNA is sufficient to rescue ventralised *ichabod* mutants (Kelly et al., 2000).

So, though it is clear that *boz* is involved in dorsal specification, there appear to be key organiser activities that do not involve *boz*. Severely affected *boz* mutant embryos have an incomplete organiser, failing to express dorsalising factors such as *chordin* and *dkk1*. These embryos also lack axial mesendoderm and show defects in anterior neural specification. Complete removal of the shield region can replicate this range of phenotypes, resulting in a loss of tissues derived from the shield region and in central nervous system (CNS) patterning defects (Saude et al., 2000). In spite of the CNS defect in both *boz* and shield ablated embryos, both the anterior-posterior (AP) and dorsal-ventral (DV) axes are specified properly, thus suggesting that *boz* is involved primarily in the specification of axial mesendoderm and that other factors are involved in specifying the organisers neural inducing and neurectodermal patterning activities.

Nieuwkoop demonstrated that a signal, which originates from the vegetal region of the embryo, is responsible for the induction of mesoderm in the cells located at the embryonic equator. This observation was exploited to identify secreted molecules that are able to act in the process of mesoderm formation. Indeed, such screens identified members of the fibroblast growth factor (FGF) family, and transforming growth factor β (TGFβ) superfamily as being able to induce mesoderm. Among these, Activin was demonstrated to be able to act as a morphogen, since it is able to induce varying mesoderm types that are dependent on the concentration of Activin (Asashima et al., 1990; Green and Smith, 1990; Smith et al., 1990). Furthermore, Activin is sufficient for the formation of dorsal mesoderm, i.e. organiser (Piepenburg et al., 2004; Smith et al., 1990). However, more recently Nodal related proteins have also been implicated as essential inducers, where both Nodals and Activin are known to operate though a common signal transduction mechanism (reviewed in (Schier and Shen, 2000)).

Loss of function studies in *X. laevis* have implicated VegT, a member of the T-box transcription factor family, in the control of initial Nodal related gene expression (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996). VegT is initially localised to the vegetal region and at the start of zygotic transcription activates zygotic signals that are vital to the correct patterning of the developing embryo. Indeed, in the absence of VegT activity there is a failure of Nodal-related growth factor expression (Kofron et al., 1999; Xanthos et al., 2001). It has also become apparent that the major targets of VegT are in fact the Nodals (Clements et al., 1999). Analysis of the promoter regions of *Xnr 1* (*Xenopus* nodal related 1) identified T-box binding sites, suggesting that VegT may act directly to up regulate the Nodals (Hyde and Old, 2000). Where there are six nodals in

Xenopus, three in zebrafish and one nodal in mouse. Although a zebrafish homologue of VegT has been identified, encoded by the *spadetail* locus, it is not expressed maternally and the phenotype resulting from loss of *spadetail* function does not produce the same range and severity as loss of VegT function in *X. laevis* (Griffin et al., 1998). However, recent work has identified the T-box protein Eomesodermin (Eom) as having an important role in organiser formation in zebrafish (Bruce et al., 2003). *Eom* is expressed in a manner resembling *VegT* expression in frog embryos, where *Eom* is expressed specifically on the dorsal side of the embryos shortly after the MBT. Removal of Eom function was noted to cause defects in organiser gene expression, with over-expression of *Eom* resulting in the formation of secondary axes. However, it was also noted that expression of zebrafish *Eom* was unable to rescue VegT depleted frog embryos, thus suggesting that though *Eom* is expressed maternally in zebrafish, much like VegT in *Xenopus*, they are not functionally equivalent leaving open the possibility that another, as yet uncharacterised T-box protein may be acting during early zebrafish development.

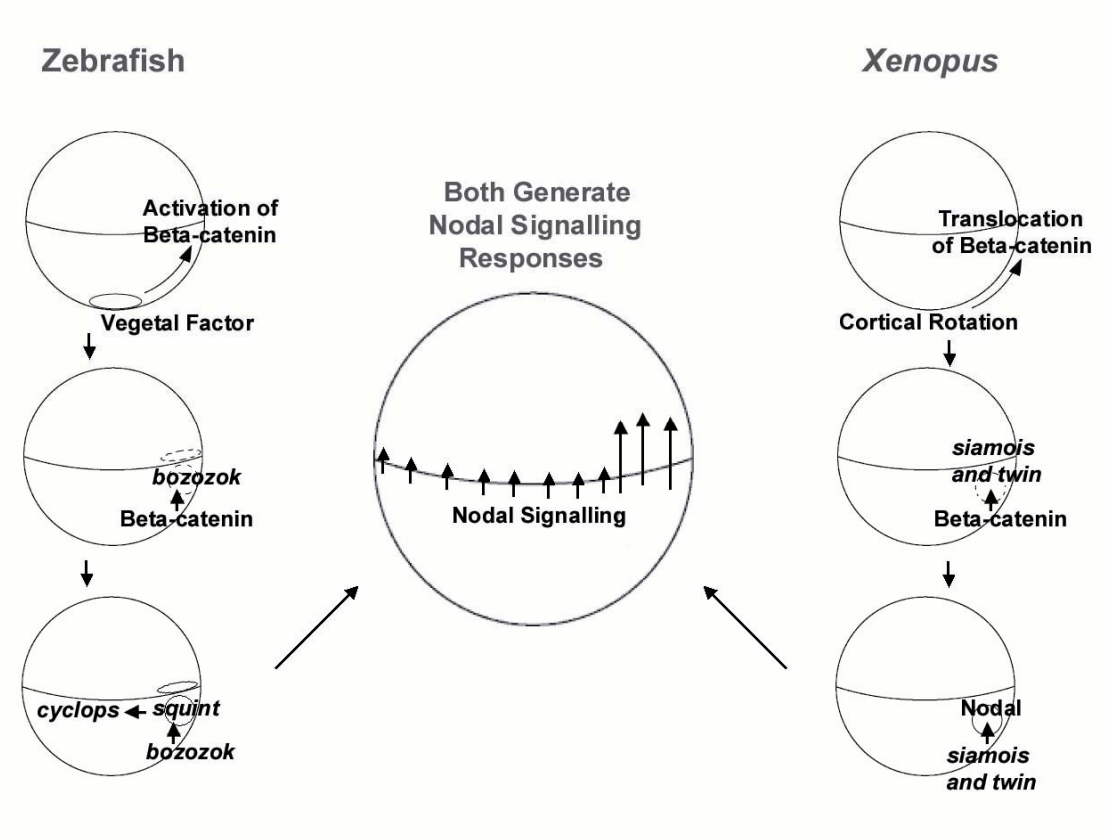
Genetic studies in mouse and zebrafish have demonstrated the essential nature of Nodals in mesoderm induction (Conlon et al., 1994; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998; Zhou et al., 1993). Two of the zebrafish nodal-related proteins, Squint and Cyclops, play essential though partially redundant roles in the specification of zebrafish mesendoderm. Double mutants for both Squint and Cyclops demonstrate a complete lack of endoderm and mesoderm, with the exception of a few somites in the tail (Feldman et al., 1998). This phenotype is replicated by maternal/zygotic (MZ) loss of the Nodal co-receptor One-eyed pinhead or the over expression of Nodal antagonists, such as Antivin/Lefty-1 (Gritsman et al., 1999; Thisse and Thisse, 1999). Fish embryos lacking *schmalspur* lack floorplate,

demonstrate reduced prechordal plate and have no medial mid or hindbrain (Brand et al., 1996). This mutation was identified in the zebrafish mutagenesis screen and encodes FoxH1, a transcription factor downstream of nodal signalling (Sirotkin et al., 2000). Despite the lack of mesoderm in Nodal mutants, embryos still possess a neuraxis with distinct anterior and posterior identities (Feldman et al., 2000; Feldman et al., 1998). Thus, at least two properties of organiser activity, neural induction and neural AP patterning, are present in the absence of the nodal derived organiser. However, ventralised embryos generated through removal of the vegetal yolk region lack not only the tissues absent in the Nodal mutants, but also the neurectoderm. Suggesting that other signals, possibly an FGF or another, as yet unidentified signal, acts to induce and pattern the neurectoderm (Reim and Brand, 2002; Streit et al., 2000).

The differentiation of mesoderm in response to nodals is complicated by the activity of mesoderm inducers of the bone morphogenic protein (BMPs) family. Several BMPs are able to induce a ventral/posterior type mesoderm (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Schmidt et al., 1995). In addition, over-expression of BMPs has been shown to prevent the formation of dorsal mesoderm (Schmidt et al., 1995). In light of this, considering several BMPs are expressed within the lateral/ventral margin, it is not unreasonable to assume that BMPs normally act as antagonists, favouring the formation of ventral/lateral mesoderm over dorsal mesoderm. Indeed, secreted inhibitors of BMPs, which include noggin, chordin and follistatin (Hemmati-Brivanlou et al., 1994; Piccolo et al., 1996; Zimmerman et al., 1996), are among the earliest dorsal-specific genes to be expressed.

In addition to members to the BMP family, Wnt signalling is also known to play an essential role in dorsal ventral patterning of the developing mesoderm (Christian et al., 1991; Christian et al., 1992). While activation of the canonical Wnt signalling pathway, involving β-catenin, will specify dorsal identity during cleavage stages, zygotic activation of the pathway will suppress organiser formation. For both the BMPs and Wnts and their antagonists, it is not clear if they have a definitive role in the establishment of organiser tissue, though they clearly are important in organiser function (Harland and Gerhart, 1997). Recent work in the zebrafish has helped define the method of Wnt8's repression of organiser formation. The transcriptional repressors Vox and Vent are direct transcriptional targets of BMP and Wnt8 signalling and embryos lacking both Vox and Vent have expanded organisers, similar to that observed in Wnt8 loss of function embryos, linking the action of Wnt8 in establishing the ventral side of the embryo directly with the up-regulation of Vox and Vent (Ramel and Lekven, 2004).

Thus, the earliest post-fertilisation events act to establish a gradient of activated, nuclear-localised β-catenin, the peak activity of which lies at the future dorsal side of the embryo. Independently of this, vegetal signals act to specify marginal, mesendodermal fates. The coincidence of high-levels of activated β-catenin with vegetally derived signals that are acting to specify mesendoderm, serves to specify the organiser as distinct from basic ventrolateral mesendoderm. At the dorsal side, high levels of Nodal activity are sufficient to specify dorsal organiser fate. In this situation Nodals are critical for mesoderm formation laterally and ventrally. Obvious comparisons can be made between this process in both *Xenopus* and Zebrafish (**Figure 1.1**).



Zebrafish:

Vegetal Factor → β-catenin → *bozozok* → Nodal

***Xenopus*:**

Cortical Rotation → β-catenin → *siamois* and *twin* → Nodal

Figure 1.1 Establishment of the dorsal-ventral axis in *Xenopus* and zebrafish.

A vegetally located factor in zebrafish and cortical rotation in *Xenopus* results in the activation of β-catenin specifically at the dorsal side in the region of YSL/Nieuwkoop centre formation. This results in the expression of *boz* in the zebrafish and *siamois* and *twin* in frog, both of which are thought to act to amplify the maternal signal, which results in the induction of nodal signalling. Nodal signalling then acts to pattern the developing mesoderm, which include the developing organiser.

1.2.2 Properties of the Dorsal Organiser

Organiser transplantation studies have revealed much about the structure and function of the organiser. Initially, work by Oppenheimer demonstrated that the *teleost* embryonic shield is the equivalent of the amphibian dorsal organiser (Oppenheimer, 1936). This has been confirmed in more recent zebrafish studies (Saude et al., 2000; Shih and Fraser, 1996). In these more recent studies, micro-dissection of organiser tissue demonstrated that the shield has separable head and trunk/tail organiser activities (Saude et al., 2000; Zoltewicz and Gerhart, 1997). In such studies, it was shown that the shield consists of a superficial epiblast layer and a deeper hypoblast layer sitting on the yolk cell, both covered with the tight-epithelial EVL. Donor tissue, dissected to enrich for deeper layer cells, was often able to induce second axes possessing anterior structures but completely lacking posterior structures, while superficial layer donor tissue was often found to induce axes consisting only of posterior structures. When the two layers are transplanted together a complete second axis was induced in the majority of experiments (Saude et al., 2000).

Expression patterns of dorsal-specific genes within the shield complement the experimental embryology. By the time the morphological shield is apparent, the expression of the homeobox genes *goosecoid* (*gsc*) and *floating head* (*flh*) is specifically restricted, since the expression of *gsc* and *flh* confined to the deep and superficial layers respectively. These regions are fated to develop into the prechordal plate and notochord (Gritsman et al., 2000; Stachel et al., 1993; Talbot et al., 1995). Prior to the formation of the embryonic shield the region fated to form prechordal plate resides close to the blastoderm margin, whereas the notochord progenitors are

situated further from the margin (Gritsman et al., 2000; Melby et al., 1996) (**Figure 1.2**). Studies on the induction of both *gsc* and *flh* in the organiser have shown that the differential activity of nodal is necessary for the correct patterning of the organiser (Gritsman et al., 2000). Over-expression of *sqt* and *cyc* induces *flh* at low doses and both *flh* and *gsc* at higher doses, demonstrating that Nodal signalling is vital for proper patterning of the organiser before gastrulation.

The defining properties of the organiser are understood primarily in the context of grafting experiments (reviewed in (Harland and Gerhart, 1997)). In such experiments, organiser tissue is capable of inducing neural development in tissue that would otherwise form non-neural ectoderm and patterns adjacent mesoderm to a dorsal fate. In searching for factors that have a role in organiser function, one successful approach has been to screen cDNA libraries to identify proteins able to induce dorsal structures in *Xenopus laevis*. Many genes identified in such a way have been found to be expressed within the organiser and have been demonstrated to have roles in the patterning activities of the organiser. Among the most abundant types of molecules identified in these screens are secreted antagonists of BMP or Wnt signalling, such as Noggin, Chordin and Follistatin, which antagonise BMP activity and prevent ventralisation. Such action promotes the development of dorsal mesoderm and neural fates (Hemmati-Brivanlou et al., 1994; Piccolo et al., 1996; Zimmerman et al., 1996). Similarly, several antagonists of Wnt signalling have suggested roles in the control of DV patterning of mesoderm and AP patterning of the ectoderm (Bradley et al., 2000; Kazanskaya et al., 2000). This growing list of molecules includes Dickkopf (Dkk1) and secreted forms of Frizzled receptors, FrzB, Crescent and Sizzled .

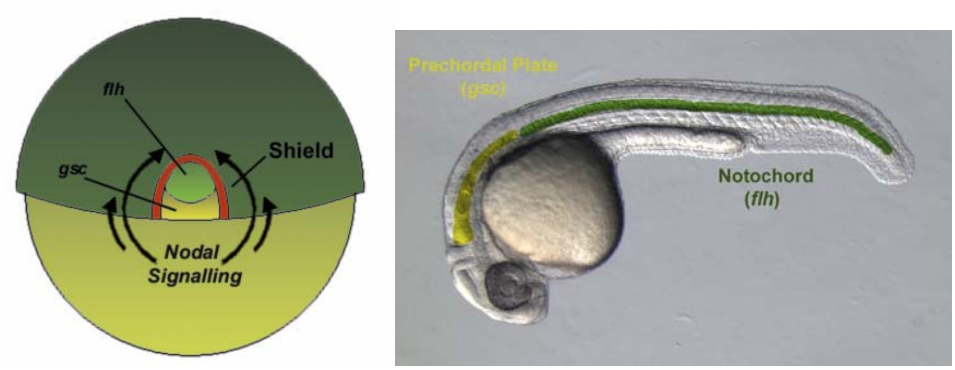


Figure 1.2 Patterning of anterior and posterior shield regions.

Left. Nodal signals pattern the organiser (shield) to form two distinct types of shield tissue, depending on the level of nodal signalling encountered. The highest levels of nodal signalling give rise to the deep, *gsc* expressing, domain, while lower levels of nodal give rise to the superficial, *flh* expressing, fated domain. These domains are fated to form the prechordal plate and the notochord respectively.

Right. In a 24-hour embryo, the prechordal plate and notochord are highlighted to show the fate of the shield regions. The deep (*gsc*) cells in yellow give rise to the prechordal plate and the superficial (*flh*) cells give rise the notochord

Genetic screens in zebrafish have also helped isolate several genes underlying the organiser's inductive capabilities. The mutants *swirl*/BMP2b, *snailhouse*/BMP7 and *somitabun*/Smad5 all encode components of the BMP signalling pathway and mutant embryos are substantially dorsalised (Hild et al., 1999; Kishimoto et al., 1997; Schmid et al., 2000). Recently, the zebrafish locus *ogon* has been found to encode Sizzled, which, similar to *Xenopus* Sizzled, was found not to inhibit Wnt8 activity but instead to modulate BMP signalling in a chordin dependent fashion, since Sizzled functions differently to Wnt inhibitors Dkk1 and Crescent and since Chordin was required for Sizzled dorsalisation (Collavin and Kirschner, 2003; Salic et al., 1997; Yabe et al., 2003). So, the model of organiser activity is one in which secreted factors that act to antagonise BMP and Wnt, establish a DV gradient within the mesoderm specifying different fates at different levels (De Robertis et al., 2001; Harland and Gerhart, 1997). While such a simple model is attractive, it does not fit several observations concerning the specification of, for example, blood, which is considered to be the most ventral mesodermal fate, though it in fact arises from nearly all regions of the mesoderm (Lane and Sheets, 2005). In addition, specification of what is considered to be the most dorsal mesoderm fate, trunk chordamesoderm, is relatively unaffected by increased or decreased levels of BMP signalling, as seen in the many zebrafish mutants that are defective in some component of BMP signalling. Thus, it appears that BMPs and zygotic Wnts act in a complicated and not yet fully understood mechanism, to pattern the established mesendoderm.

Direct ablation of organiser tissue has been achieved both genetically, as seen in *boz* mutant embryos, and surgically (Fekany et al., 1999; Saude et al., 2000; Shih and Fraser, 1996). In either case, despite the lack of organiser derived tissue,

embryos are able to develop with an essentially complete AP axis, i.e. there is a head, a spinal cord, a trunk and tail somites. Though some embryos have a partial lack of the most posterior tissue, it is clear that neural induction and patterning does occur and some somites are formed. Indicating that there is some patterning of the mesoderm. However, the removed organiser tissue is fully capable of patterning a complete secondary axis in host embryos. Thus, either the organiser, as defined by transplantation assays, is only transiently required to induce surrounding tissues, or alternatively, the organiser is a dynamic, possibly regenerative entity.

1.2.3 Specification of the three germ layers

An early process in all vertebrates is the specification of the three germ layers, where cells are specified as ectoderm, endoderm or mesoderm. It is during gastrulation that previously unspecificed cells are fated to form either the ectoderm, endoderm and mesoderm. The hypoblast, formed through the subduction of cells of the germ ring during epiboly, develops to form the endoderm and the mesoderm, with the overlying superficial layer forming the ectoderm. Work by Pieter Nieuwkoop in *Xenopus* established that a vegetal region in the egg was capable of inducing mesoderm cells in the overlying cells at the equator and that co-culture of animal cells, normally fated to become ectoderm, with this region could induce mesoderm specification (Gerhart, 1999; Nieuwkoop, 1973). As discussed previously, screens performed in the late 1980s demonstrated that Activin possesses morphogen activity (Green and Smith, 1990; Smith et al., 1990) and was shown to be sufficient for formation of organiser (Smith et al., 1990). Though it was later

established that the Nodals were the endogenous mesoderm inducers ((Jones et al., 1995), reviewed in (Kimelman and Griffin, 2000)).

VegT, discussed previously in the context of nodal signalling, is also intricately involved in the process of endoderm specification. Indeed, many endodermal genes, including *sox17, Gata5* and *Mixer*, as well as organiser specific genes are downstream of *VegT* (Xanthos et al., 2001). However, it is worth noting that TGF-β signalling is required for the proper expression of genes downstream of VegT and that a lack of VegT also results in a lack of mesoderm induction (Kofron et al., 1999). The specification of endodermal fates has also been closely linked to the specification of mesoderm. The double *sqt;cyc* and the MZ*oep* mutants that lack almost all mesoderm and also lack all endoderm (Feldman et al., 1998; Gritsman et al., 1999). Additionally, fate mapping and gene expression studies have shown that both mesoderm and endoderm arise from a bi-potent region near the vegetal margin of the developing blastoderm, termed the mesendoderm (Rodaway and Patient, 2001). However, it is not clear how mesodermal and endodermal cell fates are segregated, though it has been suggested that the timing and dose of nodal signalling are important (Aoki et al., 2002).

Further factors involved in the specification of endoderm have been characterised through analysis of endoderm mutants isolated from the zebrafish mutagenesis screen. The *casanova* locus has been shown to play an essential role in endoderm formation (Alexander et al., 1999) and has been shown by several groups to encode a novel member of the *sox* transcription factor gene family (Dickmeis et al., 2001; Kikuchi et al., 2001; Sakaguchi et al., 2001). This fits with the observation that the transcription factor Sox17 is necessary for endoderm formation in both mouse and frog (Hudson et al., 1997; Kanai-Azuma et al., 2002). The mutants

bonnie and clyde (*bon*) (Kikuchi et al., 2000), *faust* (Reiter et al., 2001), and *schmalspur* (*sur*) (Pogoda et al., 2000a), have also been shown to be defective in endoderm specification. These mutants were found to encode Mixer, a homeodomain protein, Gata5 and FoxH1 respectively (Kikuchi et al., 2000; Pogoda et al., 2000b; Reiter et al., 2001). Both FoxH1 and Mixer are required for facets of Nodal signalling in the induction of mesendoderm (Kunwar et al., 2003). The expression of *sur* is independent of Nodal, as is initial expression of *bon*, though Nodal signalling and Sur is required for its enhanced and maintained expression, suggesting, along with the observation that Smad2 associates with both FoxH1 and Mixer, a role for these factors as components of the Nodal-signalling pathway (Kunwar et al., 2003). Though the fact that complete loss of Nodal signalling results in a more severe phenotype than loss of both *sur* and *bon,* suggests that they do not compose the entire downstream pathway of Nodal-signalling. Over expression of Gata5 has been shown to lead to an expansion of endodermal cells and also induces the expression of endodermal genes in both *oep* and *bon* mutants. However, the induction of endodermal genes is less effective in *cas* mutants. Suggesting that Gata5 function downstream of *oep* and nodal, parallel to *bon* and upstream of *cas* (Reiter et al., 2001). It is perhaps unsurprising then, that homologues or both of these genes have been shown to be vital in endoderm formation in frogs (Henry and Melton, 1998; Weber et al., 2000).

1.3 Notochord

The derivative of the organiser is axial mesendoderm, which forms the hatching gland and prechordal plate in the anterior and the chordamesoderm in the

posterior. The chordamesoderm is fated to become the notochord, the defining structure of the phylum chordata. The notochord is a rod like structure, that forms early in development and serves two main roles in vertebrate development. First, as a mechanical structure, the notochord acts as the major embryonic skeletal element in lower vertebrates. Second, the notochord is essential for normal development of all vertebrates, providing signals that pattern adjacent tissues such as the gut, somites and spinal cord. Notochord development in zebrafish is relatively simple, as the notochord comprises a single cell type, surrounded by an extracellular sheath, that undergoes a characteristic series of differentiation events, marked by dramatic morphological changes. Our understanding of notochord differentiation has been significantly informed by studies of mutant zebrafish. Phenotypically, the notochord differentiation process can be broken into two discrete transitions. Firstly, the chordamesoderm is specified as a specialised mid-line mesoderm, and secondly there is a transition from chordamesoderm to notochord, which we term notochord differentiation.

1.3.1 Differentiation of the Notochord

After acting to establish the initial body pattern, the organiser differentiates and develops to form the axial mesoderm, which, in the posterior, develops into the notochord. There are two morphological features that mark the differentiation of the notochord. First, the cells of the chordamesoderm develop a thick basement membrane that forms of a sheath surrounding the notochord. Second, coupled to basement membrane formation, each cell acquires a large vacuole that acts to exert turgor pressure against the sheath. Failure to properly vacuolate leads to a

substantially shortened embryo that is easily seen in phenotypic mutagenesis screens. For this reason mutations affecting notochord differentiation are relatively easy to recognise (Odenthal et al., 1996; Stemple et al., 1996). Mutants have been identified that affect both the development of chordamesoderm and the differentiation of chordamesoderm to notochord. Analysis of these mutants has helped to reveal much concerning the processes involved in notochord development.

Identification of the mutant *flh* provided the first real insights into chordamesoderm specification. This mutation, isolated from the background of pet store zebrafish stocks, was found to encode the zebrafish homologue of the *Xenopus Xnot* gene (Talbot et al., 1995). These mutants fail to form a notochord but still form other mesoderm derivatives, such as prechordal plate and somites. In *flh* mutants, tissue that would normally form chordamesoderm instead forms somite and tissues dependent on notochord signalling, such as hypochord and floorplate, largely fail to form (Halpern et al., 1995). The gene *spadetail (spt)*, which encodes a T-box transcription factor homologous to *VegT*, is vital for embryo development and correct patterning of trunk somitic mesoderm (Griffin et al., 1998). Analysis of *flh/spt* double mutants has provided additional insight into the processes of chordamesoderm development. While *flh* mutants lack notochord, *flh*/*spt* double mutants possess trunk notochord. Thus, the *spt* mutation is able to suppress the *flh* phenotype, suggesting that *flh* acts in midline development to promote chordamesoderm and notochord fate by suppressing the induction of somatic fates in this region by *spt* (Amacher and Kimmel, 1998).

In *ntl* mutants, which lack a functional zebrafish homologue of the mouse T brachyury T-box transcription factor (Schulte-Merker et al., 1992; Schulte-Merker et al., 1994), the chordamesoderm develops normally but development arrests prior to

notochord differentiation. This contrasts *flh* mutants, in which chordamesoderm is converted to somitic mesoderm. However, the fate of chordamesoderm in *ntl* mutants is not clear. Some cells may die by apoptosis but others end up in the spinal cord and have been interpreted to form the medial floorplate, although some of these cells have been noted to express *ntl* mRNA at stages when ntl expression is normally extinguished (Stemple et al., 1996). There is also good evidence that *ntl* expression, like its counterpart in *Xenopus*, Xbra, is substantially controlled by FGF signalling (Cao et al., 2004; Griffin et al., 1998; Schulte-Merker and Smith, 1995). It is proposed in *Xenopus* that *Xbra* is involved in an indirect auto-regulatory feedback loop involving FGF. So it may be that FGF acts to maintain *ntl*, where FGF induces *ntl* expression (Cao et al., 2004; Griffin et al., 1998) and where *ntl* is able to function upstream of FGF (Casey et al., 1998; Griffin and Kimelman, 2003; Isaacs et al., 1994).

During normal development *ntl* is first expressed by marginal cells in the late blastulae and early gastrulae stages, then in internalised deep cells. Expression is then maintained only in chordamesoderm at later stages. Double mutant studies of *ntl*, *flh* and *cyc* have helped to establish the relationship between these genes in control of mid-line identities. Despite the dramatic loss of floorplate cells in *cyc* mutant embryos, double mutant *ntl/cyc* embryos display an apparent rescue of floorplate. Similarly, the majority of *ntl/flh* double mutants were found to resemble *ntl* single mutants demonstrating midline tissue not found in *flh* single mutants (Halpern et al., 1997). In the case of *ntl/flh* double mutants, since no marker of floorplate was used in the analysis, it is possible that undifferentiated chordamesoderm, which is persistently expressing early marker genes, has infiltrated the ventral neural tube. However, it is clear that midline tissue not present in *flh*

mutant embryos is present in the *ntl/flh* double mutants. While *ntl* single mutants suggest a role for *ntl* in notochord differentiation, the double mutant results show that *ntl* also has a role in chordamesoderm specification. So, considering that rescue of midline mesoderm also occurs in *spt*/*flh* double mutants and that *ntl*/*spt* double mutants have no trunk mesoderm, it appears as though ntl has some function partially overlapping with other T-box genes (Amacher et al., 2002; Amacher and Kimmel, 1998). One hypothesis is that *ntl*, *spt* and *flh* are controlling the choice between medial floorplate and chordamesoderm fate as seen with the *ntl*/*flh* double mutants, and between medial and lateral fate seen with the *spt*/*flh* double mutants and the three competing activities are balanced through feedback loops, possibly involving Nodal or FGF signalling, to ensure the appropriate amount of each tissue is specified (Griffin et al., 1995; Griffin and Kimelman, 2002; Schier et al., 1997).

Later in development, the notochord acts in the formation of vertebral bodies (centra). In zebrafish, the centra form through the secretion of bone matrix from the notochord, rather than the somites (Fleming et al., 2004; Fleming et al., 2001; Trout et al., 1982).

1.3.2 Patterning by the Notochord

The most studied signalling role of the notochord is in patterning of the neural tube. The neural tube develops distinct cell types at specific locations along its DV axis, and hence the notochord, situated just ventral to the neural tube, was considered a strong candidate for a source of patterning signals. Embryological work performed with chick demonstrated that the notochord is able to co-ordinate correct

neural tube formation, and that the absence of notochord results in abnormal formation of the neural tube (Smith and Schoenwolf, 1989; van Straaten et al., 1988). Ablation of both the notochord and the floorplate, which is itself dependent on notochord derived signals, prevents the differentiation of motor neurons and other ventral neuronal cell types in chicken as well as zebrafish (Saude et al., 2000; van Straaten and Hekking, 1991; Yamada et al., 1991). Further to this, grafting either the notochord or the floorplate to the dorsal midline of the neural tube is able to suppress dorsal neural tube fates and promote the ectopic formation of ventral neuronal cell types (Monsoro-Burq et al., 1995; Yamada et al., 1991). Similar studies have demonstrated that a diffusible signal, derived first from the notochord and then later from the floorplate, acts to pattern the neural tube (Yamada et al., 1993).

The diffusible signal involved in neural tube patterning has since been identified as Sonic hedgehog (Shh) (Echelard et al., 1993; Roelink et al., 1994). Zebrafish express three hedgehogs in the midline: *echidna hedgehog* in the chordamesoderm, *tiggywinkle hedgehog* in the floorplate and *sonic hedgehog* in both (Currie and Ingham, 1996; Ekker et al., 1995; Schauerte et al., 1998). Shh is essential for both correct patterning of the neural tube and formation of the floorplate (Ericson et al., 1996; Matise et al., 1998). It was observed, however, that ectopic Shh alone cannot induce formation of the floorplate (Patten and Placzek, 2002). However, explants of chick neural plate treated with a combination of Shh and Chordin, which is normally expressed by the notochord, develop floorplate, suggesting that the notochord produces Chordin to inhibit dorsally derived BMPs, generating a permissive environment to allow Shh to induce floorplate. The prevailing model suggests that the combination of Shh, produced ventrally, and BMPs, produced dorsally, establish opposing gradients that impart information

concerning DV position within the neural tube. *Shh* is initially expressed by notochord, then later by floorplate, with its expression being confined to the floorplate later in development.

Notochord derived hedgehog signals also have some role in both the muscle fibre type and the chevron shape that is characteristic of zebrafish somites. Normally, adaxial cells, which form immediately adjacent to the chordamesoderm and express *myoD*, will migrate to the outer surface of the developing muscle and differentiate to form slow twitch muscle fibres (Devoto et al., 1996). A few adaxial cells will eventually express Engrailed and form the muscle pioneer cells that define the horizontal myoseptum, imparting the chevron shape of the somite. When ligand activated hedgehog signalling is abolished, as in *slow-muscles-omitted* (*smu*) mutants, which lack the hedgehog signalling component Smoothened, slow twitch muscle fibres as well as the Engrailed-positive muscle pioneers fail to form (Barresi et al., 2000). Similarly, mutants lacking Shh (*sonic you)* or Gli2 (*you-too*), a transcription factor that mediates hedgehog signalling, also fail to form muscle pioneers and slow-twitch muscle fibres (Blagden et al., 1997; Karlstrom et al., 1999; Pownall et al., 1996; Xue and Xue, 1996). In notochord differentiation mutants, the somites form in an abnormal 'U' shape since the horizontal myosepta fails to form. Mutants also show compromised Engrailed expression, despite the persistent expression of midline hedgehogs in undifferentiated notochord (Odenthal et al., 1996; Stemple et al., 1996). This most likely results from a diminished capacity of the undifferentiated notochord to transmit the signal from the notochord to the forming somites (Parsons et al., 2002b).

The notochord has also been demonstrated to play a role in the development of the heart and vasculature. Removal of the anterior region of the notochord causes

an expansion of the expression domain of *Nkx2.5*, a marker for the region fated to become heart, suggesting a role for the notochord in defining the posterior limit of the heart field (Goldstein and Fishman, 1998). There is also a suggested role for the notochord in the formation of the major blood vessels of the trunk. In both *ntl* and *flh* mutants the dorsal aorta (DA) fails to form (Fouquet et al., 1997; Sumoy et al., 1997). The DA and posterior cardinal vein (PCV) form in a highly conserved fashion in vertebrates, with the DA forming just ventral to the notochord and the PCV forming dorsal to the trunk endoderm. Vascular endothelial growth factor (Vegf) is vital for the correct formation of these vessels and is thought to be sufficient for arterial specification. Over expression of Vegf in zebrafish embryos leads to ectopic expression of *ephrin-b2a*, an arterial marker, in tissue that would otherwise be venous (Lawson et al., 2002). Recent work has indicated a role for Shh in blood vessel formation. Mutants deficient in Shh were found to lack *ephrin-b2a* in the vasculature, though interestingly Vegf over-expression was sufficient to rescue arterial differentiation in the absence of Shh. In contrast, Vegf was unable to rescue arterial defects in notch signalling mutants. Taken together these data suggest a model of blood vessel formation in which Shh emitted from the notochord induces the expression of Vegf in the somites, with Vegf then acting in the DA in a Notch-signalling dependent fashion to induce proper arterial development.

The notochord also has as important role in the development of both the pancreas and the hypochord. By mechanically separating notochord from endoderm, expression of markers normally associated with pancreatic development are extinguished (Kim et al., 1997). Culture of presumptive pancreatic endoderm with the notochord induces expression of pancreatic markers, which are lacking when cultured without notochord. However, when notochord is cultured with other

endodermal tissue, pancreatic markers are not induced, suggesting that the notochord is able to induce pancreatic development only in preconditioned endoderm. The hypochord is a transient rod-like structure situated immediately ventral to the notochord and also expresses high levels *Vegf*, so may well be an important source of signals in the development of the vasculature (Cleaver and Krieg, 1998). Removal of the notochord during early neurulation stages results in a failure of hypochord formation, whereas removal of the notochord later in development does not (Cleaver et al., 2000). Thus notochord dependent hypochord induction is complete by late neurula stages. Chick transplantation studies in which notochord is grafted adjacent the endoderm have demonstrated that the ability of endoderm to form hypochord is restricted to the dorsal most region of endoderm. Moreover Notch signalling is essential for proper hypochord development (Latimer et al., 2002). Although specific roles have not been assigned, candidate notochord-derived signals controlling hypochord induction include Shh, Activin-βB and FGF2 (Hebrok et al., 1998). Finally, the notochord is vital in proper formation of the vertebral column. Removal of the notochord from both urodele and avian embryos at neural plate and 12-30 stage embryos respectively, results in a lack of proper vertebral column formation (Fleming et al., 2001).

In summary, the signalling activities of the notochord include patterning of ectoderm, specification of DV pattern in the neural tube, induction of somite, vascular and cardiac mesodermal tissues and patterning of the pancreas and hypochord endodermal tissues (see **Figure 1.3** for overview).

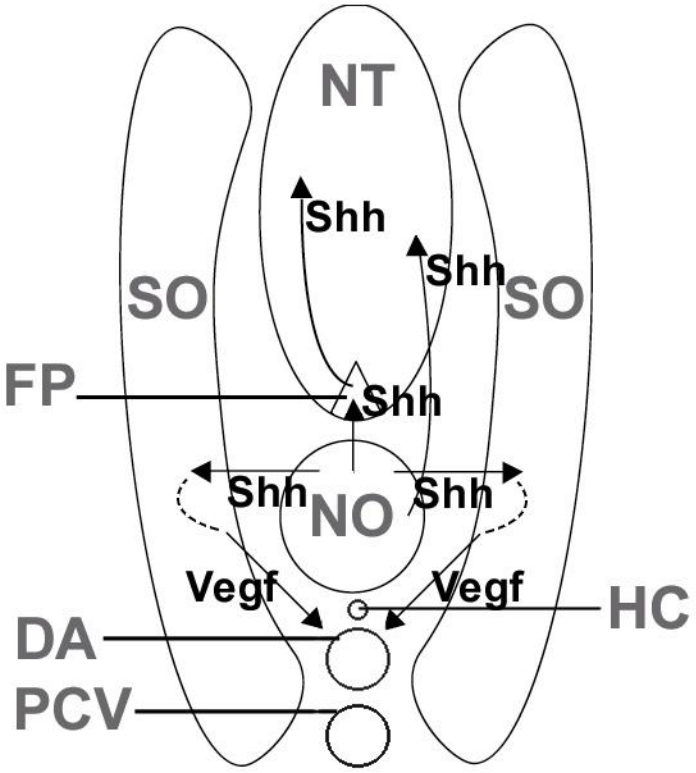


Figure 1.3 Patterning of surrounding tissues by the notochord.

Overview of the notochords role in patterning surrounding tissues; NT, neural tube; SO, somites; FP, floorplate; NO, notochord; HC, Hypochord; DA, dorsal aorta; PCV, pericardinal vein. Shh from the notochord induces floorplate and acts in early patterning of the neural tube, once floorplate is induced, shh is extinguished in the notochord and shh from the notochord patterns the neural tube along the D-V axis. Shh and Ehh (echidna hedgehog) are also involved in patterning the somites and shh signalling to the somites is able to induce vegf in the somites which then acts to pattern the dorsal aorta.

1.3.3 Mechanical Properties of the Notochord

The notochord plays a vital mechanical role in early embryos, especially for lower vertebrates where it acts as the major skeletal element, functioning in locomotion. The notochord consists of a stack of single cells, that each acquire a large vacuole surrounded by a thick sheath of basement membrane. This sheath serves as a physical boundary to limit and control the length and shape of the notochord. Turgor pressure, generated by vacuolation, is constrained by the fibrous sheath, which acts to strengthen and stiffen the notochord. In vitro experiments with *Xenopus* notochord demonstrate that notochord vacuoles will respond to environmental osmolarity, causing the notochord to lengthen and stiffen under physiological osmolarities and to become flaccid under conditions of higher osmolarity (Adams et al., 1990). The lengthening and stiffening of notochord is not observed at stages prior to sheath formation, suggesting a pathway where sheath formation must take place in order for vacuolation to proceed.

The cells of the notochord differentiate in an anterior to posterior wave, thus, the large change in cell volume of anterior cells acts to push more posterior cells caudally, causing the extension of the notochord. This extension is driven by inflation of the vacuoles constrained by the sheath, which stiffens the notochord, preventing buckling. Notochord cells are effectively "rolled" to the posterior, along the tube formed by the sheath, since strong mechanical connections, in the form of hemidesmsomes, between notochord cells and the sheath are not formed until notochord cells are mature (Coutinho, Parsons, Hirst and Stemple, unpublished observations).

The fibres of the sheath are arranged precisely and electron micrographs of transverse sections through the notochord indicate that fibres are arranged to run both parallel and perpendicular to the notochord (Parsons et al., 2002b). Studies of the precise fibre angle in the notochords of *Xenopus* embryos demonstrated that the average fibre angle within the sheath is 54°, an angle that allows the sheath to resist longitudinal and circumferential stress equally. Such as arrangement means that, provided the shape of the notochord is determined solely by inflation of the notochord cells, that the length/diameter ratio will always remain constant (Adams et al., 1990).

The structure of the notochord also functions to constrain in the type of tail movements an early embryo can make. If the notochord consisted only of a thick sheath filled with vacuolated cells it would be able to bend in any direction. However, the notochord is coupled to two other structures that mechanically serve as restraints. Dorsal to the notochord is the floorplate, which expresses many of the same extracellular proteins as the notochord, including α1 Collagen Type II (Yan et al., 1995). Ventral to the notochord is the hypochord, which also expresses similar proteins. These two structures serve as cables, running along the dosal and ventral side of the notochord, limiting its movement. Thus any force exerted on the notochord by surrounding muscle can only result in a left-right movement of the tail, due to the lateral positioning of the somites. Such motion is consistent with the requirements for forward locomotion in the early embryo.

Hence, cells of the notochord act, via vacuolation, to generate a sufficient force to support the embryo. Cells enlarge and exert pressure on the thick sheath of basement membrane that surrounds the notochord generating a hydrostatic force

similar to the turgor pressure of plant supportive networks. This inflation also acts to elongate the embryo, since an absence of inflation leads to a dramatic reduction along the AP axis. The inflation, which begins at the anterior end of the notochord and proceeds towards the posterior, effectively pushes posterior cells towards the posterior end as they expand, where these cells then expand and exert the same force on their neighbouring cells, resulting in a general extension of the embryos along the AP axis.

1.4 Notochord Mutants

In October of 1980 a paper published by C. Nusslein-Volhard and E. Wieschaus reported the first systematic search for genes involved in early development in *Drosphilia* (Nusslein-Volhard and Wieschaus, 1980). The genes identified in this systematic screen have revolutionised our understanding of animal development. Orthologous genes to those originally identified in *Drosphilia* have been uncovered in essentially every other species of animal and have helped demonstrate a remarkable conservation of developmental mechanisms throughout evolution.

Soon after this screen was published, G. Streisinger proposed that a similar screen could be performed with relative ease using the zebrafish (Streisinger et al., 1981). Just over a decade later Christianne Nusslein-Volhard and Wolfgang Driever initiated just such large-scale mutagenesis screens, for recessive-zygotic mutations in the zebrafish. Although much had been learned concerning metazoan development from the fly screen, unique developmental processes, including those involved in

development of the notochord and neural crest, could only be dissected through studies of vertebrate developmental genetics. The results of the zebrafish screen, performed in two parts, in Boston and in Tübingen, were published in 1996 and gained a great deal of publicity (Eisen, 1996)(Development **123**, 1996).

The loci identified by this screen have provided developmental biologists with an incredible resource with which to piece together the molecular mechanisms involved in early vertebrate development (Felsenfeld, 1996; Granato and Nusslein-Volhard, 1996; Holder and McMahon, 1996). The completion of this screen has prompted many groups to perform further smaller-scale screens, more focussed on specific processes (Patton and Zon, 2001).

The screen generated and isolated many mutations that resulted in notochord defects. From the Boston screen, 65 mutations corresponding to 29 complementation groups were identified with four loci identified in Tübingen (Odenthal et al., 1996; Stemple et al., 1996). These mutants demonstrate defects in notochord specification, differentiation, degeneration, maintenance and shape. Many of these mutants have a characteristic shortening of the body axis due to a lack of extension along the AP axis. Mutants were divided into several classes according to the type of observed phenotype. Two of these notochord mutants, *floating head* and *no tail*, had been isolated previously and are defective in chordamesoderm specification and differentiation into notochord respectively.

As well as *no tail*, eight other mutants were identified as defective in the differentiation of chordamesoderm to notochord, as demonstrated by the maintenance of early notochord markers such as *collagen type II* and *shh*. Of these eight mutants,seven were named after the seven dwarves *sneezy* (*sny*), *dopey* (*dop*),

happy (*hap*), *doc*, *bashful* (*bal*), *grumpy* (*gup*) and *sleepy* (*sly*), because of the stark reduction of the AP axis.

1.4.1 Dwarf Mutants

The "seven dwarves" have been grouped into three classes based on their phenotype and analysis of these mutants has revealed much about the process involved in the development and differentiation of notochord. Characterisation of these mutants has also revealed a startling similarity in the components affected in each class.

In the first class, *bal*, *gup* and *sly*, were all identified with a large number of alleles. These mutants are grouped together based on their shared lack of notochord differentiation, as demonstrated by maintained expression of early notochord markers and their shared brain defects (Odenthal et al., 1996; Stemple et al., 1996). In *bal, gup* and *sly* the brain has an irregular morphology, the hindbrain ventricle is enlarged and the axonal scaffold is disrupted (Schier et al., 1996). It is also noteworthy that all three of these mutants were also identified in a screen for retino-tectal path finding mutants (Karlstrom et al., 1996). The mutants *gup* and *sly* have the same overall morphological defects, with all *bal* alleles identified showing a much weaker phenotype than either *gup* or *sly*. The *bal* mutants fail to develop notochord in the anterior but demonstrate the same eye and brain defects as *gup* and *sly* and contain large amounts of apparently WT notochord caudally.

Cloning of *bal, gup* and *sly* identified the three mutants as the α1, β1 and γ1 laminin chains respectively (Parsons et al., 2002b; Pollard, 2002). These three specific laminin chains interact to form the Laminin-1 chain, an essential component of the notochord basement membrane sheath. Loss of laminin α1, β1 or γ1 leads to a dramatic reduction in the levels of Laminin-1 throughout the embryo, thus preventing the formation of the basement membrane surrounding the notochord. The lack of basement membrane results in a notochord differentiation defect. Loss of laminin β1 or γ1 results in a complete failure to form the notochord basement membrane. Loss of laminin α1 in *bal* mutants does not affect posterior notochord basement membrane and hence the posterior notochord is able to differentiate normally. Recent work has demonstrated that this posterior differentiation is due to the ability of laminin α4, which interacts with laminin β1 and γ1 to form Laminin-8, to act in concert with laminin α1 to form notochord basement membrane. One possibility is that laminin-α1 is acting to form basement membrane along the anterior notochord while laminin α4 contributes to the basement membrane in the posterior notochord (Pollard, 2002). Lack of both laminin α1 and α4 results in a phenotype comparable to that of *gup* and *sly*, where there is a complete lack of notochord differentiation. Hence, Laminin isoforms can act interchangeably in forming the basement membrane of the notochord.

In the second class of mutants, *dopey* (*dop*), *happy* (*hap*) and *sneezy* (*sny*) are grouped together based on their near identical phenotype. All three mutants show a similar failure in notochord differentiation, maintaining the expression of early markers, and have disrupted notochord sheath formation, similar to the *bal, gup* and *sly* mutants. However, *dop, hap* and *sny* also exhibit a marked reduction in pigmentation and show widespread degeneration by 48 hpf (Coutinho et al., 2004).

Recent work, including some performed as part of this thesis, has identified *dop, hap* and *sny* loci as coatomer (COP) β', β and α respectively ((Coutinho et al., 2004), this thesis). These are all subunits of the seven subunit COPI complex, which is involved in retrograde transport in the secretory pathway and maintenance of the composition of processing vesicles involved in secretion (Letourneur et al., 1994; Orci et al., 1997; Schmid, 1997). COPI vesicles are required for proper secretion, hence *dop, hap* and *sly* mutants h.ave a compromised secretory network, which results in defective notochord basement membrane sheath formation and defective notochord differentiation. The specific developmental phenotype observed in *dop, hap* and *sly* is reinforced by the observation that, although COPα is ubiquitously expressed, it is specifically up-regulated in chordamesoderm cells. This supports the observation that, as with the mutants *bal, gup* and *sly*, there is a link between formation of the basement membrane sheath and differentiation of the notochord. Perhaps most interestingly, work on the COPI mutants has demonstrated that expression of the COPα subunit may well be regulated in some way by the demand for secretion and COPI activity.

The final class of notochord differentiation mutations comprises *doc* and *ntl*, which fail to form fully differentiated notochord, leading to the persistent expression of some early markers and a failure in formation of the vacuoles. However, unlike the laminin and COPI mutants, *doc* and *ntl* mutants possess normal basement membrane. Transplantation experiments have shown that the notochord differentiation defect is cell-autonomous for both *ntl* and *doc* (Halpern et al., 1993; Odenthal et al., 1996). Of these two loci, *doc* has the most notochord-specific defect. Though *ntl* mutants fail to generate tails, in the trunk region they are phenotypically very similar to *doc* mutants. The *doc* mutants however, demonstrate defects only in

notochord differentiation, which in turn leads to defects in the patterning of surrounding tissues. This is the case in all the notochord mutants and is likely to be due to a lack of signalling from a properly differentiated notochord. A detailed understanding of the upstream factors controlling *doc* and *ntl* should elucidate the nature of the notochord differentiation signal and an understanding of their downstream effectors should reveal further insights into how differentiation is manifest. One of the primary aims of this thesis is to clone the *doc* locus and to determine how this gene product interacts with the known process of notochord development to control and coordinate differentiation. The presence of a normal basement membrane sheath surround the notochord in *doc* mutants suggests a possible role for *doc* in the final stages of notochord differentiation that take place only after the basement membrane has formed (see **Figure 1.4**).

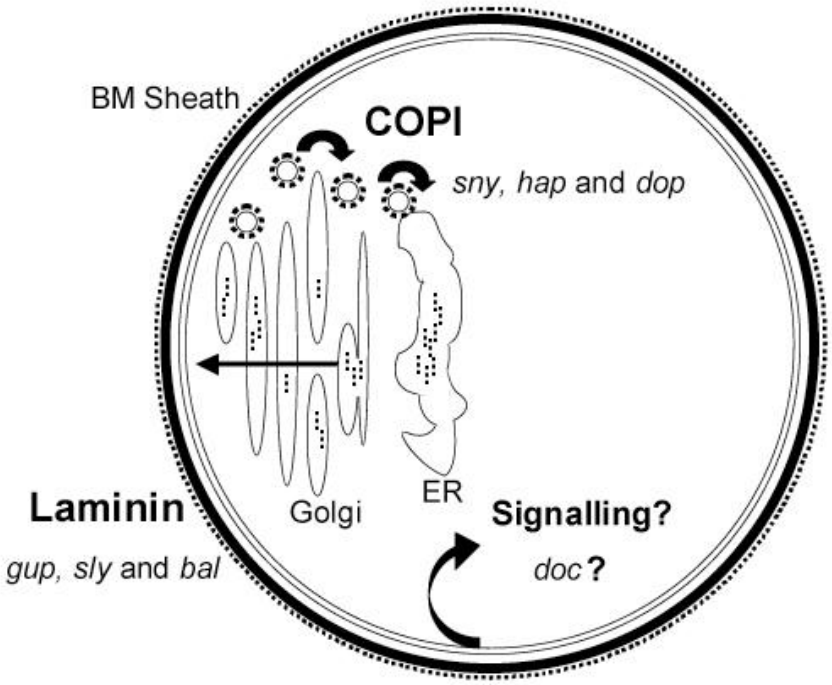


Figure 1.4 Roles of notochord dwarf mutants in notochord development.

The mutants *gup, sly* and *bal* encode the laminin chains β1, γ1 and α1 lack vital components of the notochord BM sheath. The mutants *sny, hap* and *dop* lack the COPI subunits α, β and β'and are defective in retrograde transport, causing defects in the secretory pathway. The mutant *doc* may well be defective in signalling important for notochord differentiation, possibly relating information about BM sheath formation back to notochord cells.

1.5 Notochord Sheath

The cells of multicellular organisms are surrounded and supported by the extracellular matrix (ECM), which can be essectially described as secreted molecules that are immobilised outside cells and can be broadly said to consist of three classes of molecules: collagens, non-collagenous glycoproteins and proteoglycans. The ECM is capable of affecting many cellular processes in an instructive manner (Adams and Watt, 1993) and during embryogenesis, populations of cells undergo many morphogenetic events that involve direct cell-ECM interactions (Zagris, 2001). Noteably, Epithelial cells form sheets and tubes, neural crest cells migrate large distances and cell-ECM interactions affecting cell migration have been characterised.

One important property of the ECM arises through the formation of a specialised type of matrix known as basement membrane (BM). Many proteins including fibronectin, collagen and laminin have been shown to make up this matrix, which is essential in early vertebrate development.

The BM can control many aspects of cell/tissue behaviour during development and following injury (Schwarzbauer, 1999). Investigations into the properties of BM have demonstrated that laminin, which is a major constituent of BM, is a mediator of ductal or tubular morphogenesis and differentiation (Edwards et al., 1998; Jiang et al., 1999; Schuger, 1997; Streuli et al., 1991; Thomas and Dziadek, 1994). BM also acts in the kidney glomerulus as an important component of the selective barrier that prevents passage of macromolecules from the blood into the urine and is known to have an important role in localisation of the synapse in the

neuromuscular junction both during embryogenesis and after injury (Carbonetto and Lindenbaum, 1995; Sanes and Lichtman, 1999).

1.5.1 Components of the Notochord Basement Membrane

The properties of the BM are a direct result of the properties of its component parts. BM consists primarily of laminin, which is cross-linked to type IV collagen by entactin or nidogen and includes proteoglycans such as aggrecan.

The laminins are a family of heterotrimeric glycoproteins and are one of the earliest extracellular matrix proteins secreted during development (reviewed in (Colognato and Yurchenco, 2000)). Three polypeptide chains, α, β and γ, make up the laminin heterotrimer complex. To date, five α, four β and three γ genes have so far been identified in mouse. Combinations of these proteins give rise to the multiple laminin isoforms, though there appears to be restrictions so that only a subset of all possible combinations are produced. Currently twelve isoforms have been reported. The Laminin heterotrimer is formed through interactions between the coiled-coil domains in the C-terminus, known as the long arm and the N-terminus of each chain contains, which contains globular domains, gives rise to the short arms. The globular domains within the N-termini of α chains are the major sites of interaction with cell-surface receptors such as integrins. As well as binding sites for collagen IV and nidogen, laminins are also able bind to each other and hence form large BM networks (Tunggal et al., 2000).

Laminin 1 (α1β1γ1) was identified in 1979 in extracts from the Englebreth-Holm-Swarm (EHS) murine tumour and teratocarcinoma cells (Timpl et al., 1979) and is the most well characterised of the Laminins. Laminin 1 appears to be the main laminin involved in early development and was the first to be completely sequenced and structurally analysed. Many of the domains responsible for the various ligand interactions were identified using purified Laminin 1. The roles of various Laminins *in vivo* have been better characterised through genetic studies of certain human diseases and targeted gene disruption in mice, reviewed in (Colognato and Yurchenco, 2000). Characterization of these phenotypes has revealed an unexpected diversity of function, demonstrating roles in processes as diverse as cell migration, differentiation, metabolism and polarity (Colognato and Yurchenco, 2000; Gustafsson and Fassler, 2000).

1.5.2 Formation of the Notochord Basement Membrane

Analysis of the notochord mutants has demonstrated the importance of BM formation in notochord development and has also provided much information on the processes involved in the formation of the basement membrane sheath, demonstrating what tissues are involved in the establishment of the notochord sheath. Transplantation studies in sheath mutants, where the shield from either a mutant or wild-type embryo is transplanted onto a wild-type or mutant host respectively, have shown much. In such studies a secondary notochord of the donor genotype is generated that is completely surrounded by tissues of the host genotype, allowing the origin of laminin components of the basement membrane to be dissected. Such

transplantation studies performed with the *bal, gup* and *sly* mutants have demonstrated that the laminin chains could be supplied either by the notochord or by non-notochordal tissues, since both transplantation of mutant shields onto wild-type hosts and wild-type shield onto mutant host leads to embryos that have proper notochord differentiation. Hence, the notochord basement membrane sheath can be supplied both autonomously and non-autonomously.

Although the Laminin rich layer of the notochord basement membrane can be supplied by either the notochord or the surrounding tissues examination of three other notochord differentiation mutants, *dop, hap* and *sny* demonstrated that this was not the case for the medial layer of the sheath. An absence of Laminin, which contributes greatly to the inner layer of the sheath, causes an absence of organised basement membrane sheath. However, in *dop, hap* and *sny*, the inner, Laminin rich layer, still forms but there is still an absence of organised notochord basement membrane. Transplantation experiments, to examine if the medial and outer layers were notochord autonomous or non-autonomous, established that the establishment of the medial and outer layers of the sheath are notochord autonomous. Shields transplanted from *sny* embryos onto wild-type hosts resulted in secondary notochords with disrupted sheaths lacking the medial layer, whereas wild-type shields transplanted into *sny* mutant hosts, generated secondary notochords with wild-type sheaths. So, it can be said that Copα, and most probably the entire COPI complex and secretory system, acts autonomously within the chordamesoderm/notochord to ensure formation of proper medial layer basement membrane formation and thus ensure notochord development.

1.6 Summary of Thesis Results

The results of this thesis are presented in five chapters, with the sixth chapter providing a discussion of results and an overview of the future perspectives raised by this work. In the first chapter, I describe the work performed to define the *doc* locus to a specific genomic location and identify candidate genes within this region. In the second chapter, the work undertaken to characterise which candidate is responsible for *doc* is described. In this chapter, a novel multiple WD40 domain protein is proposed as *doc* and evidence to support this is described. In chapter three, the work in characterising the *dop* and *hap* loci is discussed and evidence that they encode the COPI subunits COPβ' and COPβ is provided. Following that, the characterisation of the remaining COPI subunits is covered and evidence for an auto-regulatory mechanism in COPI subunit expression is discussed. The following chapter, chapter four, describes the work undertaken to uncover the regulatory mechanism involved in COPI expression. In the next chapter, a brief examination of the effect of COPI loss of function on ER and Golgi structure is reviewed. Following this, chapter seven describes the work undertaken to uncover the regulatory mechanism for COPI and the UPR is put forward as an essential regulatory mechanism required for proper development. The discussion describes the arguments based on the results of this thesis and discusses the future directions suggested by the work described herein.

Chapter Two

Materials and Methods

2 Materials and Methods

2.1 Embryo collection

Zebrafish (*Danio rerio*) female and male pairs were placed in tanks together in the evening before eggs were required. Eggs are usually laid and fertilised the following morning shortly after the lights are turned on (lights on occurs regularly at 8:30am), though on occasion when embryo collection was desired later, males were kept physically separated from the females within the same tank and released when eggs were required. Embryos were collected in Embryo Water (red sea salt 180mg/l, methylene blue 2mg/l) shortly after having been laid. Embryos were raised from the day of collection up to 5 days at 28°C in Embryo Water. Embryos were staged according to the morphological criteria provided in (Kimmel et al., 1995). Zebrafish embryos collected for staining procedures were fixed at least overnight in 4% PFA in phosphate buffered saline (PBS: 137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4.7H_2O$, 1.4mM KH_2PO_4) at 4ºC. Embryos of 24 hpf or older were de-chorionated prior to fixation, to prevent fixation of the embryos with curled trunks, whereas embryos younger than 24 hpf were de-chorionated after fixation and before dehydration. Following fixation, embryos were dehydrated in increasing concentrations of MeOH in PBS (25%, 50%, 75% then 100%). Dehydrated embryos were stored in 100% methanol at -20 °C until required.

2.2 General molecular biology techniques

2.2.1 Small scale preparation of DNA

The Qiagen Spin miniprep kit (Quiagen) was used for all small-scale plasmid preparations, according to the manufacturer's protocol.

DNA and RNA were quantified by spectrophotometry at 260 nm (an OD of 1 was equated to 50 μg/ml double stranded DNA, 35 μg/ml single stranded DNA and 40 μg/ml RNA). The ratio between the readings at 260 nm and 280 nm provided an estimate of the purity of the nucleic acid preparation (pure preparations of DNA and RNA should have OD_{260}/OD_{280} values of 1.8 and 2.0, respectively).

2.2.2 Gel extraction of DNA

For the extraction of DNA from agarose gels, the QIAquick Gel Extraction Kit (Qiagen) was used according to manufacturers protocol. Samples were eluted in 30μl of water, 4μl of this was used for TOPO cloning or standard ligation reactions and 5μl was used in a standard 50μl PCR reaction.

2.2.3 Phenol/Chloroform extraction

To remove proteins from nucleic acid solutions, a mixture of phenol:chloroform:isoamyl-alcohol (25:24:1 volume ration) was added in a 1:1 volume ratio to the DNA solution and shaken for 1 minute. The sample was then centrifuged for 5 minutes and the upper (aqueous) layer was transferred into a new microcentrifuge tube, a further extracted with an equal volume of chloroform was performed to remove traces of phenol.

2.2.4 Ethanol Precipitation

Ethanol precipitation was carried out by adding 3 M NaOAc pH 5.5 (to a final concentration of 0.3 M) and 3 volumes of ice-cold 100% ethanol to the DNA

solution, which was then left on dry ice for approximately 20 minutes. Centrifugation at 20,000g for 5 to 20 minutes was performed and the DNA pellet was then washed in 70% ethanol, dried and re-suspended in TE or distilled water.

2.2.5 TOPO cloning

The cloning of PCR products was performed using the TOPO TA Cloning®kit (Invitrogen). The cloning reaction was performed according to the following conditions: 4 µl fresh PCR product or 4µl of gel purified product, 1µl of 1.2M NaCl solution and 0.5 µl pCR®-TOPO® vector. These were mixed gently and incubated for 5 minutes at room temperature.

2.2.6 Transformation of chemically competent bacteria

Transformation of the ligated vector was performed using chemically competent TOP10 cells (Invitrogen). Briefly, 2µl of TOPO ligation mix was added to 25µl cells and incubated on ice for 30mins. Cells were then heat shocked for 30s at 42^{o}C, then immediately transferred to ice, incubated for 2mins before adding 250µl SOC added. Cells were then incubated at 37^{o}C for 1hr and an aliquot of 10 to 200 µl from each transformation was spread onto a selective agar plate (100 mg/ml of ampicillin) and incubated overnight at 37°C. 40 µl of X-Gal (20 mg/ml in dimethylformimide) and 40 µl of IPTG (200 mg/ml) were used per plate for selection.

2.2.7 Restriction digestions

Restriction enzyme digests were performed at the recommended temperature for approximately 2 hours using commercially supplied restriction enzymes and buffers (Boehringer Mannheim, Promega, New England Biolabs). The enzyme

component of the reaction never comprised more than 10% of the reaction volume. For enzyme digests using more than one restriction enzyme, the buffer suggested by the manufacturer was used.

2.2.8 DNA Sequencing

DNA sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit, according to the manufacturers instructions, in an ABI 377 automatic sequencer.

2.3 Bioinformatics and Genomics

DNA sequence manipulation and analysis was performed with either Sequencher, DNAStrider or DNASTAR software. Protein alignments were performed with the Clustal method in MegAlign (DNASTAR). Open reading frames were examined using ORFinder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

2.3.1 Identification of zebrafish orthologues of mouse or human proteins

Zebrafish ESTs corresponding to orthologues of yeast, mouse or human genes were sought either by name in the nucleotide databases or by probing the zebrafish EST database with a defined protein sequence using the tblastn algorithm at http://www.ncbi.nlm.gov/BLAST/. Sequences found were clustered using Sequencher or SeqMan software. When required, ESTs were ordered to obtain DNA templates for riboprobe synthesis (from the Integrated Molecular Analysis of

Genomes and their Expression, IMAGE or Resource Centre/Primary Database, RZPD).

To define further sequence beyond EST data, the zebrafish genomic database was searched at http://www.ensembl.org/Multi/blastview?species=Danio_rerio. In all cases, consensus sequences were used to probe the protein databases using the blastx algorithm, at http://www.ncbi.nlm.gov/BLAST/, to confirm gene identity (Altschul et al., 1990).

2.3.2 Analysis of Identified Genes

The mRNA and protein sequence of genes identified from genomic sequence were examined using appropriate blast programmes to identify homologues. Zebrafish EST data was probed with the full length protein sequence of identified homologues using tblastn to extend zebrafish sequence and provide full length sequence data. Sequence data was clustered using SeqMan and protein sequence compared to homologues using MegAlign.

2.3.3 Oligonucleotide design

PCR primers were designed using the program Primer3 (Whitehead Web Page) at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi, from regions where reliable DNA sequence was available (Rozen and Skaletsky, 2000).

To disrupt mRNA translation, MOs were designed to target the region around the ATG start of translation. In general, the translation start site of zebrafish genes was identified by alignment of orthologues protein sequences belonging to several species and by examination with ORFinder. Splice MOs were designed against

splice sites identified through analysis of genomic and cDNA sequence. Sequence encompassing the target was sent to Gene Tools LLC, which designed the MO to be purchased. All MOs were 25-mers of approximately 50% G/C content, with less than 36% G content and no more than two consecutive Gs, and forming no more than 4 contiguous internal base pairing. Each design sequence was tested for representation elsewhere in the genome. The MOs designed for this work are listed in **Table 2.1**.

Targeted gene	MO sequence (5'-3')
Control	CCTCTTACCTCAGTTACATTTATA
IRE1	AGCAAACCAGCAACAGCAGCATCTG
XBP1	GTCCCTGCTGTAACTACGACCATTT
ATF-6	AACATTAAATTCGACGACATTGTGC
PERK	CTCCGTCCAGAGAGGGAATGAACAT
BiP	GCAAAAACAGGCAAAGCAACCGCAT
Cadherin-13	CTGGTCCAGTAAGTCATTGTGACAC
doc 1	GAGTTCCACTTGAAAGAAATGTCAT
doc 2	TGTGTCTTCTGCCATCGTGATACTT

Table 2.1 MOs used in this thesis.

2.4 Preparation of genomic DNA from Adult Fish

Whole tail fin from adult fish was dissected and placed in 0.5ml of extraction buffer (0.5% SDS, 0.1 M EDTA pH.8.0, 10mM Tris pH.8, 100 µg/ml Proteinase K) for 5 hours at 55°C. Phenol chloroform extraction and ethanol precipitation were carried out, and the final pellet was resuspended in 30µl TE. A 1:100 dilution of this was used PCR template. 5µl of 10-50 ng/µl adult fish DNA was used in a 20µl reaction.

2.5 Preparation of genomic DNA from embryos

Embryos were dehydrated in methanol, placed in individual wells of 96-well plates and the methanol evaporated. The digestion of each single embryo was done by incubating in 100 µl 100 µg/ml Proteinase K for 5 hours at 55ºC and the Proteinase K denatured by incubation at 95ºC for 5 minutes. 1µl of this crude solution was then used in a 20µl PCR reaction.

2.6 Polymerase Chain Reaction

2.6.1 SSLP mapping

All PCR reactions were performed in 96-well plates with a final reaction volume of 20 µl. For a 20µl total volume PCR reaction we used 4µl of 5x PCR buffer (0.25M KCl, 50mM Tris pH8.4, 12.5mM $MgCl_2$, dNTPs at 1mM and BSA 0.85µg/ml), 2µl 10mM forward and reverse primer solution, 0.2 µl of Taq polymerase (5u/µl) and an appropriate volume of template was used. Sterile H_2O was used to make the reaction volume up to 20µl. Plates were covered with

Microseal 'A' Film (Cat# MSA-5001, MJ Research, Inc) and sealed with heated lid PCR machines. The PCR conditions were as follows:

94ºC for 3 min

35 cycles:

92ºC for 30secs

62ºC for 30secs

72ºC for 30secs

72ºC for 10 min

This PCR program was also used for all RH mapping and for various PCR amplifications, though the extension time was varied accordingly where 1kb of DNA was allowed 1minute extension at 72ºC. On occasion, PCR reactions were amplified across a gradient range (55ºC-70ºC) of primer binding temperatures to determine optimum conditions.

2.6.2 Radiation hybrid panels

96 PCR reactions were performed using the LN54 panel (Hukriede et al., 1999). 20µl total reaction using 0.2µl of AmpliTaq Gold (Perkin Elmer), 2µl of each primer (10µM), and 4µl PCR buffer (5X). The SSLP PCR program was used (see previous section). Analysis of the results and the calculation of linkage and LOD scores was carried out at http://mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi.

2.6.3 dCAPS Analysis

Primers for dCAPS analysis were designed using the web-based dCAPS finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) with wild type and mutant genomic sequence for COPβ and COPβ' (Neff et al., 2002). The primers used for PCR were:

COPβ forward: 5'-GTTGAGAATGAGTTGAAGAAGGAGGCTCGA-3'

COPβ reverse: 5'-GAAACGTCAGTGTGGACGTG-3'

Which generated an XhoI polymorphism in the wild type sequence.

COPβ' forward: 5'-AGGCAGCCTTTCTGCCCGCACAGA-3'

COPβ' reverse: 5'-CGCACTGCTCTCTAGGGTTT-3'

Which generated an XhoII polymorphism in the wild type sequence.

2.6.4 RT-PCR

For total RNA extraction, embryos were pooled and homogenized in 0.5ml of TRIzol reagent (GibcoBRL) and left at 37ºC for 5mins. 100µl of chloroform was then added, samples were mixed by hand for 15s and then centrifuged at 13000rpm for 10mins at 4^{o}C. The aqueous phase was transferred to a fresh tube and RNA was precipitated with isopropyl alcohol, and then centrifuged for 30mins. The resulting pellet was washed with 70% ethanol and re-suspended in DEPC treated water and the concentration of RNA determined. First strand cDNA was synthesized using superscript reverse transcriptase (GibcoBRL) as manufacturers instructions. Briefly, 1µg of total RNA was used in a 20µl reaction with either random hexamer, polyT or gene specific primers, cDNA was amplified for 1hour at 42ºC and diluted 1:10. 5µl of this template solution was used in a 50µl PCR reaction. On occasion, large fragments or fragments cloned for sequencing were amplified using the KOD Hot Start kit (Novagen) as per the suppliers directions.

2.6.5 Rapid amplification of cDNA ends (RACE)

RACE was performed using the GeneRacer cDNA amplification kit (Invitrogen). mRNA was isolated from total RNA using TRIzol (as described above). The synthesis of the cDNA modified with the Generacer 5' and 3' adaptors was performed as per the manufacturer's protocol, using either random primers or poly-T primer in the reverse transcription reaction. All RACE reactions were performed by nested PCR. The GeneRacer 5' primer (CGACTGGAGCACGAGGACACTGA) or the GeneRacer 3' primer (GCTGTCAACGATACGCTACGTAACG), when performing 5' or 3' RACE reactions, respectively, and a gene-specific primer (GSP 1) designed using Primer3 software (see above), were used in a first round of PCR, for 15 cycles. The product of this round of PCR was diluted 1/40 and 5 µl of this dilution was used as template for the second round of PCR. In the latter, the GeneRacer 5' nested primer (GGACACTGACATGGACTGAAGGAGTA) or the GeneRacer 3' nested primer (CGCTACGTAACGGCATGACAGTG), when performing 5' or 3' reactions, respectively, and a second nested gene-specific primer (GSP 2) were used for 25 cycles. The Touchdown PCR program was used in all cases with the extension time varying according to the size of the expected product (2min per kb).

94°C for 3 minutes

5 cycles:

94°C for 30s

72°C for 4 mins*

5 cycles:

94°C for 30s

70°C for 4 mins*

25 cycles:

94°C for 20s

68°C for 4 mins*

*(extension time in min was varied according to the expected length of the cDNA; e.g. 1kb = 2mins, 2kb = 4min etc.).

2.6.6 Agarose Gel Electrophoresis

Analysis of polymorphic markers in meiotic mapping and examination of nucleic acid size was performed by agarose gel electrophoresis. Gels were prepared by dissolving agarose in 1x TAE (20 mM TRIS acetate, 1 mM $Na_2EDTA.2H_2O$ (pH 8.5)) to a final concentration of 3-4% for polymorphic analysis and 0.8–2% (w/v) for size determination, depending on the expected size of the DNA fragments, and 0.4% ethidium bromide. Nucleic acid samples were mixed with 5x gel loading buffer (Orange G: 4g Sucrose and 0.025g Orange G in 10ml water) and, in the case of RNA, with RNase inhibitor. Electrophoresis was performed at 5–20 V/cm gel length until appropriate resolution was achieved. Ethidium bromide-stained nucleic acid was visualised using ultraviolet light ($\lambda \approx 302$ nm) and fragment size was estimated by comparison with the 1kb or 100b ladder molecular weight markers (Promega) run in at least one of the gel lanes.

2.7 Whole-mount *in situ* hybridisation

For the synthesis of riboprobes, template (plasmid) DNA was linearised for 2 hours, phenol/chloroform purified, ethanol precipitated and then resuspended. Approximately 2μg of linearised plasmid was used in the synthesis of probe. In all cases, digoxigenin (DIG)-labelled uracil triphosphate (UTP) (Boehringer Mannheim)

was incorporated during RNA transcription, as per the manufacturer's instructions. After synthesis, riboprobes were treated with 20 U DNase I (Boehringer Mannheim) at 37 °C for 15 min to remove DNA template and were purified by size-exclusion chromatography through a DEPC water column (Clontech Chroma Spin-100). All riboprobes were electrophoresed on a 1% agarose gel to check size and integrity prior to use. Riboprobes were added to Hyb shortly after synthesis and were stored at -20°C. **Table 2.3** has a list of all cDNAs used as templates for anti-sense RNA probes used in this work, as well as the respective origin.

Whole-mount *in situ* hybridisations were performed essentially as described by Thisse (Thisse et al., 1993). Embryos fixed with 4% paraformaldehyde/PBS at 4°C were dehydrated with methanol at -20°C and rehydrated by soaking for 5 minutes each in 75% methanol/PBT (1x PBS + 0.1% Tween 20); 50% methanol/PBT; 25% methanol/PBT and then 4 times 5 minutes in 100% PBT. All embryos >36 hpf were digested with proteinase K (10 µg/ml) for 5 minutes and then refixed in 4% paraformaldehyde for 20 minutes at room temperature and then washed in PBT 5 times 5 minutes. They were then transferred to hybridisation buffer (50% formamide, 5X SSC (pH7.0), 500 µg/ml type VI torula yeast RNA, 50 µg/ml heparin, 0.1% Tween 20, 9 mM citric acid to pH 6.0-6.5) for 2-5 hours at 70°C (prehybridisation). The hybridisation buffer (Hyb) was then replaced with the mixture containing 150 ng of DIG-labelled RNA probe in 200 µl of preheated hybridisation solution and the embryos were incubated at 70°C overnight. Washes were performed at the hybridisation temperature with preheated solutions for 15 minutes each with 75% Hyb/2X SSC; 50% Hyb/2X SSC; 25% Hyb/2X SSC; 100% SSC and finally 2 times 30 minutes in 0.2X SSC. A series of washes were performed at room temperature for 10 minutes each in 75% 0.2X SSC/PBT; 50%

0.2X SSC/PBT; 25% 0.2X SSC/PBT and 100% PBT. Embryos were blocked in 2 mg/ml BSA, 2% goat serum in PBT for several hours and then incubated with alkaline-phosphatase (AP)-conjugated anti-DIG Fab fragments diluted 1:5000 in 2 mg/ml BSA, 2% goat serum in PBT at 4°C overnight with agitation. After washing at least 8 times for 15 minutes with PBT, the embryos were rinsed 3 times 5 minutes in NTMT reaction buffer (0.1 M Tris-HCl pH9.5; 50 mM $MgCl_2$; 0.1 M NaCl; 0.1% Tween 20). Detection was performed using NBT/BCIP (112.5 µl of 100 mg/ml NBT in 70% dimethylformamide and 175 µl of 100 mg/ml BCIP in 70% of dimethylformamide 1added to 50 ml of NTMT). After stopping the reaction with 100% PBS (pH 5.5), the embryos were refixed in 4% paraformaldehyde/PBS. Embryos were cleared with 20% glycerol/80% PBS, 50% glycerol/50% PBS and stored at 4°C in 80% glycerol/20%PBS.

2.8 Morpholino injection

Morpholino oligonucleotides (MOs) were obtained from Gene Tools, LLC. MOs work through an RNase-H independent process, via binding directly to target RNA sequence to either block translational initiation at the ATG or prevent correct splicing. Control MO was that suggested by Gene Tools, LLC.

Lyophilised MO was re-suspended in 60µl of deionised water. 1µl of this solution was diluted in 799µl 0.1NHCl and O.D measured at 265nm. Precise concentration was determined the specific absorbance and molecular weight as provided by Gene Tools. Prior to microinjection, the MOs were diluted using MO buffer to titrate the dose (5 mM Hepes pH7.2, 0.2 M KCl and 2.5 mg/ml phenol red).

A volume of 1.4nl was injected through the chorion and into the yolk of 1 to 4-cell stage embryos to deliver the desired dose of MO.

Injection needles were prepared by pulling filament-containing borosilicate glass capillaries (Word Precision Instruments, 1B100F-4, outside diameter 1.0mm, inside diameter 0.75mm) with a vertical pipette puller (David Kopf Instruments), cutting the edge with a razor blade, and calibrating under the microscope with a millimetre ruler. Injection system consisted of a needle holder (Word Precision Instruments), carried by a 3-axis micromanipulator (Narishige), connected to a compressed air flow controlled by a control panel (World Precision Instruments) and triggered by a foot pedal. Zebrafish embryos were injected at the 1 to 4-cell stage with 1.4 nl of the desired solution. Embryos were aligned on the side of a glass slide in a sterilin Petri dish under relatively dry conditions.

2.9 Confocal Microscopy

Calreticulin-CFP (Cal-CFP) and 1,4-galactosyltransferase-YFP (GalT-YFP) plasmids were obtained from BD Biosciences (Clontech Living Colors pECFP-ER Cat. #6907-1 and pEYFP-Golgi Cat. #6909-1). The plasmids were linearised with StuI and Co-injected into 1-cell stage embryos in a volume of 1.4nl at a concentration of 10ng/µl each. These embryos were injected with either 3.5ng COPα MO or 3.5ng of control MO in a 1.4nl volume. Confocal microscopy was performed on BioRad Radiance 2100 system with a Nikon E800 Eclipse microscope.

Embryos used for confocal microscopy were collected, injected and incubated in 0.3x Danieau's solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$,

0.6 mM $Ca(NO_3)_2$, 5 mM HEPES (pH 7.6)) to prevent any residual fluorescence from the methyl blue in zebrafish blue water.

2.10 Photomicrography

High-power images from both live and fixed embryos were obtained using a Zeiss Axiophot microscope fitted with either a Kodak DCS420 digital camera, or a Jenoptik Jena system that used Openlab 3.1.2 software. Living zebrafish embryos were photographed in 3% methylcellulose (Sigma). Images were treated with AdobePhotoshop.

Solution	Formulation
1X PBS	137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4
1X PBT	1X PBS, 0.1% Tween 20
1X TAE	40 mM Tris.Acetate, 2 mM $Na_2EDTA.2H_2O$ (pH 8.5)
1X TE	1 mM EDTA, 10 mM Tris.HCl pH 8.0
20X SSC	3 M NaCl, 0.3 M Na_3citrate.$2H_2O$, adjust pH to 7.0 with 1 M HCl
5x PCR buffer	0.25M KCl, 50mM Tris pH8.4, 12.5mM $MgCl_2$, dDTPs (A, T, G, C) at 1mM and BSA 0.85µg/ml
1x Danieau's solution	58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES (pH 7.6)
Orange G (5x)	4g Sucrose, 0.025g Orange G in 10ml H_2O

Table 2.2 Formulation of frequently used solutions.

Protein	Origin	Enzyme	Pol
COPβ	RT-PCR product (this thesis)	EcoRV	SP6
COPβ'	RT-PCR product (this thesis)	BamHI	T7
COPε	RT-PCR product (this thesis)	XbaI	SP6
COPδ	RT-PCR product (this thesis)	XhoI	SP6
COPγ2	RT-PCR product (this thesis)	HindIII	SP6
COPζ	RT-PCR product (this thesis)	HindIII	T7
COPζ2	RT-PCR product (this thesis)	EcoRV	SP6
Echidna hedgehog	(Currie and Ingham, 1996)	EcoRI	SP6
BiP	RT-PCR product (this thesis)	SalI	SP6
doc	RT-PCR product (this thesis)	HindIII	T7

Table 2.3 In situ hybridisation probes used in this thesis.

Chapter Three

Positional Cloning of *doc*

3 Positional Cloning of *doc*

In this chapter the techniques used to identify the gene responsible for a mutant phenotype will be discussed. The methods required to determine a genes position and then identify the gene within that region responsible for a mutant phenotype will be covered. This introduction will also provide examples of the application of this approach to successfully identify mutant genes in the zebrafish. The results of the positional cloning of *doc* are then presented.

3.1 Introduction

There are two dominant approaches used to clone the gene responsible for a specific mutant phenotype; the candidate gene approach and positional cloning. The candidate gene approach has been used successfully on number of occasions to identify mutated zebrafish genes. This approach uses functional information, obtained from previous work, to suggest genes that may act in a way that could bring about the mutant phenotype. Two examples of this approach are the zebrafish mutants *no tail* and *floating head*, which were found to be homologues of the *Xenopus* genes *Xbra* and *Xnot* respectively (Schulte-Merker et al., 1994; Talbot et al., 1995). However, this approach is limited to well studied processes and genes, and in cases where there is no obvious candidate, such as is the case when isolating novel genes, cloning via the identification of the genes position in the genome becomes necessary (Talbot and Schier, 1999). This approach was initially developed as a means of identifying the genes responsible for human genetic diseases (Collins

et al., 1992) and in 1986 was demonstrated to be a functional approach to identifying mutations with the identification of the gene responsible for chronic granulomatous disease (Royer-Pokora et al., 1986). Since then, many inherited human diseases have had the gene responsible identified through the application of positional cloning techniques. These include Duchenne muscular dystrophy, cystic fibrosis, fragile X syndrome and breast cancer (Fu et al., 1991; Kerem et al., 1989; Miki et al., 1994; Monaco et al., 1986; Riordan et al., 1989; Rommens et al., 1989; Verkerk et al., 1991). This method has become more powerful and accessible in recent years through the availability of high quality genome sequence. The sequencing of whole genomes in human, mouse and, most importantly in terms of this thesis, zebrafish has dramatically decreased the work necessary to identify a gene based on its genetic location.

Positional cloning in zebrafish, or recombinant mapping, relies on the use of polymorphisms that occur between the mutant strain and the mapping strain, where the mapping strain is crossed with the mutant carrier to generate a mapping line from which mutant and wild type sibling embryos are collected. The identification of polymorphic markers that are closely linked to the mutation, through comparisons of wild type and mutant embryos, is a critical step in any positional cloning project. If such flanking markers can be found then the amount of work involved later in chromosome walks and candidate identification is greatly reduced. Hence, the availability of dense genetic maps is highly desirable. For zebrafish, the first genetic map was based on random amplified polymorphic DNA (RAPD) (Postlethwait et al., 1994) and identified 401 loci, which was increased to 652 by 1996 (Johnson et al., 1996). However, genetic mapping using RAPD analysis has many drawbacks and thus short sequence length polymorphism (SSLP) markers have become the preferred

marker type for genetic mapping studies (Beier, 1998). SSLPs utilise PCR primers that flank short microsatellite repeats, typically the dinucleotide cytosine-adenine (CA), that are spread throughout the genome and that vary in the number of repeats. A map based on SSLP marker was published for the zebrafish in 1996 and characterised 102 SSLPs. Since then further markers have been added and the total number now exceeds 2000 (Shimoda et al., 1999). However, one drawback of SSLP maps is that a high proportion of markers will not be polymorphic between two specific strains and so are not useful for mapping. Thus, any marker that might be useful must initially be tested to determine whether it is polymorphic between the mapping lines.

The positional cloning approach has been used to successfully identify many zebrafish genes, including novel genes (Zhang et al., 1998), genes that provide functional models for human disease (Parsons et al., 2002a), genes that highlight the conserved nature of development (Kikuchi et al., 2001) and, most relevantly to this thesis, genes involved in development of the notochord (Coutinho et al., 2004; Parsons et al., 2002b) (**Table 3.1**). Positional cloning is a labour intensive process and utilises a multitude of genetic tools to identify the gene responsible. Since the publication of the large-scale ENU mutagenesis zebrafish screens in 1996 such tools have become a source of much development in zebrafish, steadily increasing the ease with which mutations can be mapped. Currently, there is a relatively comprehensive SSLP map, containing thousands of markers spread throughout the genome (Postlethwait et al., 1994; Shimoda et al., 1999), which has been supported recently by genome sequencing. The generation of large scale genome sequence has allowed markers to be more accurately positioned and also offers up identified insertion/deletion (INDEL) fragments and BAC end fragments as alternate markers

for use in fine mapping. Radiation hybrid (RH) maps, large-insert genomic libraries and the large number of published expressed sequence tags (ESTs) (reviewed in (Beier, 1998)), also aid in positional cloning. Further to these tools, zebrafish genome sequence, which is currently estimated at more than 1.5Gb of sequence, allows for the rapid and easy identification of candidate genes and gene sequence that, when coupled with the ability to easily test gene function through 'knock-down' with anti-sense morpholino oligonucleotides (MOs), enables the rapid identification and testing of candidate genes once genomic location has been identified. Such techniques have succeeded in making positional cloning a straightforward means to characterise a genes identity from the identification of mutant phenotypes (Fishman, 1999; Talbot and Schier, 1999).

Mutant	Protein	Reference
casanova	Sox related protein	(Kikuchi et al., 2001)
gridlock	Hairy-related bHLH	(Zhong et al., 2000)
heart and soul	PKC lambda	(Horne-Badovinac et al., 2001)
miles apart	Sphingosine-1-phosphate receptor	(Kupperman et al., 2000)
one-eyed pinhead	EGF-related protein	(Zhang et al., 1998)
pickwick	titin	(Xu et al., 2002)
ogon	Sizzled	(Yabe et al., 2003)
grumpy	laminin β1	(Parsons et al., 2002b)
sleepy	laminin γ1	(Parsons et al., 2002b)
bashful	laminin α1	(Pollard, 2002)
sneezy	COPα	(Coutinho et al., 2004)

Table 3.1 Examples of Zebrafish genes identified by positional cloning.

one-eyed pinhead was the first zebrafish gene identified by positional cloning. Positional cloning has been used to identify four of the seven zebrafish dwarf mutants.

Positional cloning is performed in a series of steps where the mutation is defined to smaller and smaller genomic regions. The primary step in positionally cloning any gene is to define the mutation to a specific linkage group. There are several methods that can be used in this step, including gynogenic half-tetrad diploids (Johnson et al., 1995) and bulk segregant analysis (BSA), which involves the identification of polymorphic markers that are linked to a mutation by studying the segregation of markers between pools of WT and mutant embryo DNA (Beier, 1998). Once linkage to a specific linkage group has been confirmed, genetic mapping is performed to find linked markers on either side of the mutation, with markers within that region then tested to define to mutation to regions of decreasing size. Once known genetic markers are exhausted, the genomic sequence can be used to design new markers, which can be tested for polymorphisms between the two mapping strains.

The zebrafish is an excellent system in which to perform meiotic mapping studies, as large numbers of meioses can be analysed with relative ease. This then increases the probability of identifying useful crossovers and enables the mutation to be mapped with greater resolution to a genomic region. By crossing two genetically distinct strains of zebrafish fish, one of which contains the mutation and the other of wild-type phenotype, hybrid fish are produced that can then be interbred to produce embryos suitable for mapping. By using these embryos, genetic markers can be used to perform meiotic mapping, where the frequency of recombination between the marker and the genetic locus is measured through the use of markers that vary between the two strains. Approximately one in three known SSLP markers vary between any two zebrafish strains. The recombination events of these polymorphism markers between the mapping strains are then used identify a closely linked marker,

or, ideally, two closely linked markers that flank the mutation on either side. This involves genotyping many phenotypically mutant fish, in an attempt to identify recombination events that assist in the determination of the distance and order of markers. Thus, a candidate interval is defined, which is reduced through further identification of new markers and continued mapping. Once all known polymorphic markers are exhausted, further markers can be designed from genomic sequence to further resolve the genomic region. By comparing INDELs between the two mapping strains, polymorphic markers can be identified that can be further used to refine the mapping region. Once the mutation has been defined to a suitable region sequence either from the genome sequencing project or, in cases where this sequence is uncertain, from self generated sequence from BACs can be analysed to identify candidate genes. Once candidates are identified there are three well-established techniques that can be used to test and prioritise them: the attempted rescue of the mutant phenotype, through over-expression of cRNA or DNA (Yan et al., 1998), though this is not always straight forward, due to complications of gene mis-expression; the expression of the candidates can be tested through *in situ* hybridisation, to examine if the gene is expressed in a manner befitting the phenotype; and the function of a gene can be examined through targeted gene knock-down with MOs in an attempt to 'phenocopy' the mutant (Nasevicius and Ekker, 2000). Once a suitable candidate is identified, the mutation itself can be characterised through cloning and sequencing of wild type and mutant cDNA.

In recent years, the zebrafish has become a far more versatile system for studying developmental biology through the use of the MO gene knock down technique, which provides a quick, easy and relatively cheap method of removing a gene to study its function. MOs are short oligonucleotides that possess a morpholino

ring, rather than a ribose sugar and phosphoamidite rather than phosphodiesters. This abnormal backbone means that MOs are more resistant to degradation than conventional oligonucleotides and are nevertheless capable of hybridising with endogenous nucleic acids. Mos have proved to be particularly effective in the study of developmental biology in a number of organisms and has rapidly become a popular and powerful tool (Heasman, 2002; Heasman et al., 2000). The phenotypes of *no tail*, *chordino* and *one-eyed pinhead* have all been successfully phenocopied using MOs (Nasevicius and Ekker, 2000). Moreover, all of the identified zebrafish notochord mutants have had their respective phenotypes copied using MOs, demonstrating that these phenotypes are easily replicated with this technology (Coutinho et al., 2004; Parsons et al., 2002b). MOs are generally designed to prevent translation through hybridisation to the endogenous mRNA at either the start of translation, which knocks down both maternal and zygotic transcripts, or at a splice sites in the un-edited RNA (Draper et al., 2001), which knocks down only the zygotic transcript. MOs are stable (operating via steric blocking), cheap, and extremely effective. Production of a given mutant phenotype after injection of a MO against a particular candidate gene provides strong evidence that it represents the mutated gene.

3.2 Initial Mapping of *doc*

Initial mapping of *doc* involved the linking of the mutation to a specific chromosome. Pools of 48 mutant and 48 wild type sibling (which would include homozygous wild type as well as heterozygous embryos) were used to test linkage of

doc to two sets of SSLP markers spaced throughout the genome. Each panel consisted of 192 markers. Markers were amplified from both mutant and wild type pools, then products run on 3% agarose to examine linkage of polymorphic markers. Using this method, non-mendelian inheritance of the marker D3 on plate H2-2 was noted. This lack of recombination between the mutant locus and the marker Z9484 linked *doc* to LG18. (**Figure 3.1**)

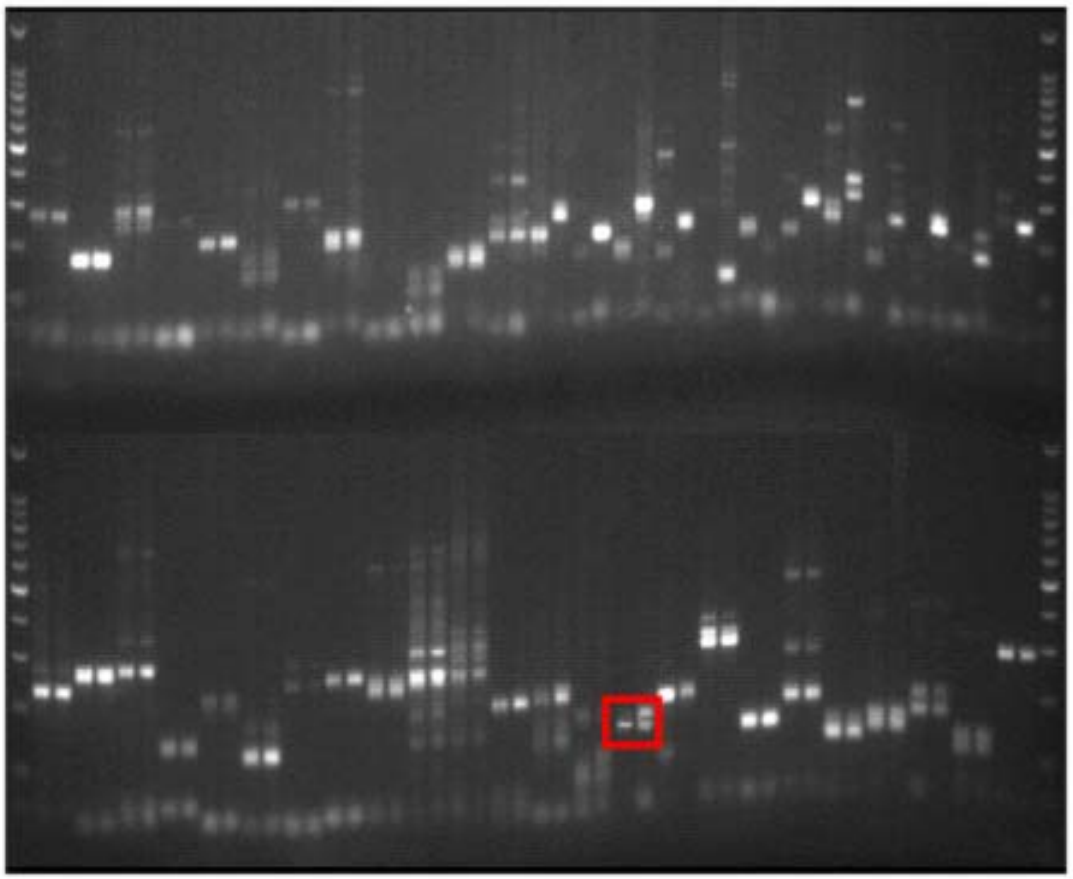


Figure 3.1 Likage group mapping of *doc.*

Mapping panel H2-2, marker a1-12 and b1-12 (top row) and c1-12 and d1-12 (bottom row). PCR products of mutant pool (left) and wild type sibling pool (right) run in tandem. Bordered in red is marker d3 (Z9484), mutant pool shows one marker product where the wild type sibling pool shows both polymorphic products. Products run on 3% agarose, 100bp ladder (promega #G210A) run on the left and right of the gel.

3.3 Confirmation of Linkage and Further mapping

3.3.1 Confirmation of Linkage to LG18

To confirm linkage of *doc* to linkage group 18, three polymorphic markers, Z9484, Z7654 and Z7417 were identified using the MGH zebrafish microsatellite map (Shimoda et al., 1999) (http://zebrafish.mgh.harvard.edu/). Products were amplified from 48 single *doc* mutant fish and 48 single wild type siblings and run on 3% agarose to examine recombination events. Linkage was demonstrated to all three markers, with the 48 mutant embryos showing four recombination events between *doc* and marker Z9484, 10 recombination events between *doc* and marker Z7654 and four recombination events between *doc* and marker Z7417 demonstrating a distance of 4.1cM, 10.4cM and 4.1cM from the markers respectively.

3.3.2 Defining a Region for *doc*

By comparing the occurrence of recombination in specific individuals, markers can be placed on either side of the mutant locus, as recombination occurs only once on a single linkage groups during meiosis in zebrafish (Streisinger et al., 1986). All four recombinant embryos between *doc* and marker Z9484 were unique, whereas the four recombinants between Z7417 and *doc* were also recombinant for Z7654. Thus, the markers Z7417 and Z7654 flank one side of *doc* with Z9484 flanking the other side. The two closest markers to *doc* on either side were identified as Z9484 and Z7417. Using publicly available genome sequence

(http://www.ensembl.org/Danio_rerio/), this placed *doc* in a region of ~12Mbases on linkage group 18.

3.3.3 Fine mapping of *doc*

Using publicly available genome sequence, BAC end sequence and INDELS (insertion/deletion sequences) were identified and primers designed to amplify ~200bp regions to test for polymorphisms. Initially, 20 BAC ends and 18 INDEL markers were tested, spaced approximately evenly across the region. One of the BAC end markers and two of the INDEL markers were polymorphic between the mapping strains. Testing of the BAC end marker with the 48 mutant embryos identified that one of the embryos demonstrating recombination between Z9484 and *doc* was also recombinant. Thus, this marker, at roughly the 11.1Mb position on LG18 is positioned between Z9484 and *doc*. Mapping of the INDEL markers enabled finer positioning of *doc*. Both of the polymorphic markers demonstrated recombination within one the mutant embryos that demonstrated recombination for Z7417, demonstrating that they are positioned between *doc* and Z7417. The closest of these markers to *doc* was located at approximately the 11.5Mb position on LG18. Thus, *doc* is situated between the two identified polymorphic markers in a region of ~0.5Mbases between 11.1 and 11.5 Mb on LG18.

3.4 Identifying Candidates

Using SSLP and INDEL markers, the *doc* locus was defined to a region of ~0.5Mbases on linkage group 18. Using publicly available genomic sequence, this region was examined for identified or predicted genes that might play a role in notochord development. Three such genes were identified: Cadherin-13, Syntaxin-8 and a novel multiple WD40 domain protein.

3.5 Discussion

Through the use SSLP markers covering the entire zebrafish genome, linkage of *doc* to linkage group 18 was established, since the panel marker Z9484 showed no mendelian inheritance in pooled mutant samples. This linkage was confirmed through the further use of SSLP markers, including Z9484 and known linked markers from the genetic map. These markers also assisted in defining the *doc* locus to a specific region, since the recombinants for Z9484 and the markers Z7417 and Z7654 were discrete individuals. Thus, since recombination occurs only once on each linkage group during meiosis in zebrafish, the markers were considered to be situated on either side of the mutation, as no mutant embryo demonstrated recombination for both Z9484 and Z7417 and/or Z7654. Since the frequency of recombination between *doc* and Z7417 was less than the frequency of recombination between *doc* and Z7654, *doc* was characterised as lying between Z9484 and Z7417.

The genetic map provided no polymorphic markers closer to *doc* than Z9484 and Z7417, which provided a genomic distance of ~12Mb in which the mutation was situated. Such a region was far too large to attempt to find candidate genes from available sequence and so genomic sequence was instead used to try to provide further markers for recombinant mapping. INDELs are commonly identified

polymorphisms in the zebrafish genome, resulting from the insertion or deletion of small fragments of non-coding genome between strains. These INDELs were used to design and identify polymorphic markers for use in further mapping. Using such markers, mapping of *doc* was further refined and the mutation was mapped to a region of ~0.5Mbases. Within this region, three possible candidate gene were identified from the genomic sequence (see **Figure 1.1** for an overview of mapping).

3.6 Summary

- *doc* is located on linkage group 18.
- *doc* lies between the markers Z9484 and Z7417, in a defined region of ~0.5Mbases.
- Within this region, three gene were identified as likely candidates.

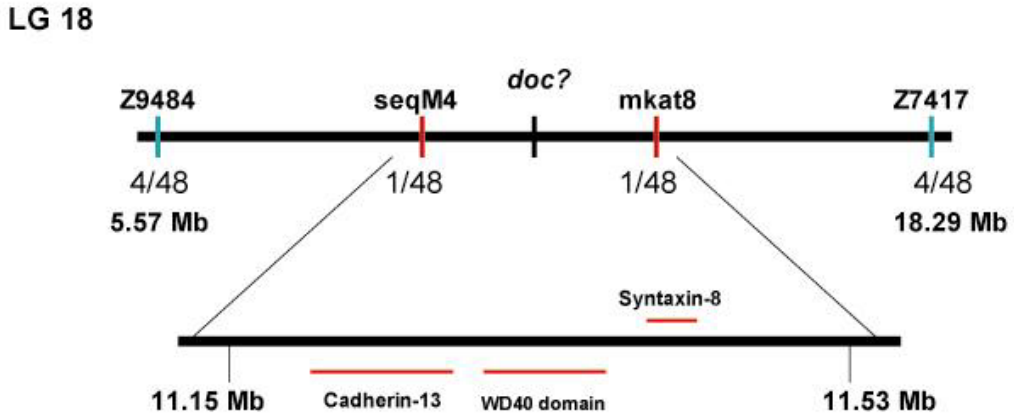


Figure 3.2 Overview of mapping of *doc*

Chapter Four

Characterisation of *doc*

4 Characterisation of *doc*

4.1 Introduction

In the previous chapter, the work performed to define the *doc* locus to a genomic region was described. Within this region, three candidate genes were identified: a novel gene, containing multiple WD40 domains, Cadherin-13 and Syntaxin-8. In this chapter, the work undertaken to define which of these genes is responsible for the *doc* phenotype is described, including MO knockdown of mRNA and *insitu* hybridisation to examine the expression profile of genes.

4.2 MO Knock-Down of Candidate Genes

Using previous observations concerning the gene size of cloned mutants and the number of identified alleles of the mutations, from the positional cloning of *sny, gup, sly* and *bal* (Coutelle et al., 2001; Parsons et al., 2002b; Pollard, 2002), the likelihood of each candidate encoding *doc* was examined according to the size of the candidate gene and the observed number of *doc* alleles. The outcome of such consideration was that Cadherin-13 and the multiple WD40 domain protein were considerably more likely to encode *doc* than syntaxin-8. As such, MO's designed against the ATG of the ensemble predicted genes were designed and used to examine the role of each gene in development.

MO's against both the ATG of Cadherin-13 and multiple WD40 domain protein (doc 1) were injected at a concentration of 8ng and 4ng in a volume of 1.4nl into embryos staged between fertilisation and 4-cell stage.

Injection of either 8ng or 4ng of Cadherin-13 MO resulted in no discernable phenotype at 24 hpf. However, injection of 4ng and 8ng of doc 1 MO resulted in a marked reduction of the A-P axis, defects in somite development, with somite blocks forming in a 'U' shape as opposed to the normal 'V' shape and a loss of proper notochord development. Thus, initial evidence suggested that the WD40 domain protein encoded *doc* (**Figure 4.1**).

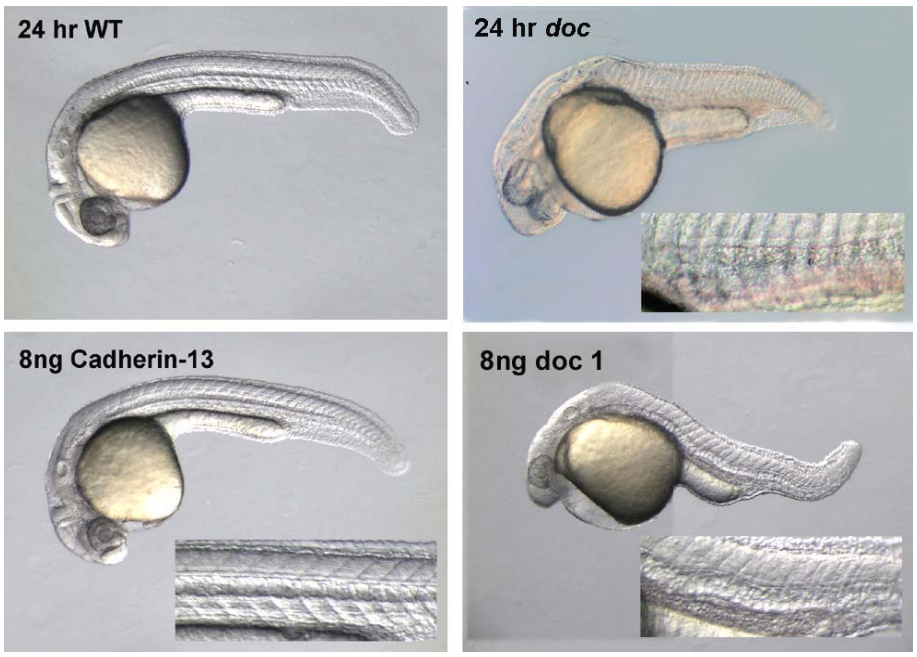


Figure 4.1 MO knock-down of candidate genes.

Lateral view, anterior to the left, ventral to the top, of live 24 hpf embryos. 8ng of Cad-13 shows no observable phenotype as 24 hpf. 8ng doc 1 shows mild phenocopy of *doc* (top right), with a lack of A-P extension, loss of notochord differentiation and 'U'-shaped somites. Boxes show enlarged notochords.

4.3 Analysis of the Novel Multiple WD40 Domain Gene

The ensemble predicted novel WD40 domain gene encoded a cDNA of 1654 bases, which in turn encoded a predicted protein of 447 amino acids. tblastn analysis of the zebrafish gene identified a novel *X. laevis* gene with considerable homology. Comparison of this gene to the zebrafish predicted cDNA demonstrated that the ensemble predicted sequence lacked approximately 500 bases of 5' sequence. By blasting the *X. laevis* protein sequence of this gene against the zebrafish EST database, additional sequence was identified. Compiling of this sequence generated a 2194 base cDNA encoding a protein sequence of 617 amino acids. Analysis of this sequence using Blastp, identified homologous proteins throughout evolution. Related mouse, human and *X. laevis* sequences showed 65%, 64% and 67% identity respectively. **Figure 4.2**.

Figure 4.2 Comparison of doc homologues

Zebrafish, *X. laevis*, Mouse and Human homologues of doc. Shaded bases differ from the zebrafish sequence. Compiled using seqman.

4.4 Additional MO Analysis of doc

Identification of the additional 5' sequence for the novel, WD40 domain protein generated a new initiation ATG, raising queries concerning the previously observed MO result. Analysis of the initial MO suggested that it was binding to an internal region within the mRNA, with the possibility that is was binding to a splice site. As such, a new start of translation ATG targeted MO (doc 2) was designed and injected at 8ng and 4ng in a volume of 1.4nl into embryos staged between fertilisation and 4-cell stage.

The observed phenotype for this new MO was highly similar to the previous phenotype, with embryos displaying shortened A-P axis, abnormal 'U' shaped somites and a lack of distinct notochord development. The only obvious difference between the doc 1 MO and the doc 2 MO was the severity of the phenotype. At 4ng, doc 1 produced a mild phenotype, with defects only becoming obvious at 8ng, whereas the doc 2 MO resulted in an obvious phenotype at 4ng, which became more severe at 8ng (**Figure 4.3**). This secondary doc MO, which binds to the start of translation, as opposed to the internal binding of doc 1 MO, results in a more severe phenotype at lower doses. Despite this, both MOs result in a lack of notochord differentiation, a reduction of AP extension and a loss of proper patterning of surrounding tissues by the notochord.

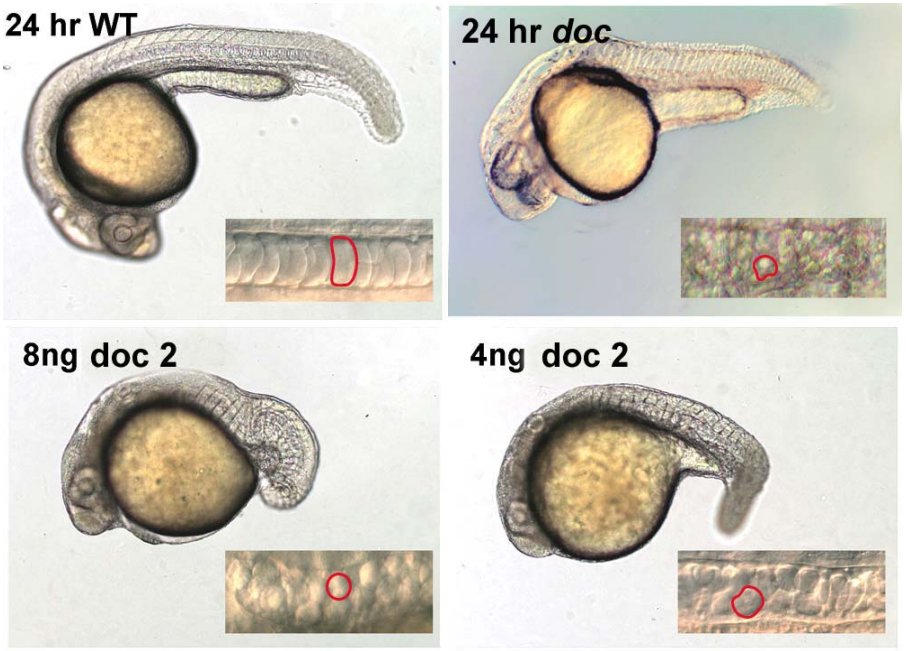


Figure 4.3 MO knock-down with secondary doc MO.

Lateral view, anterior to the left, ventral to the top, of live 24 hpf embryos. Secondary MO knock-down of the WD40 domain protein more accurately phenocopies *doc*. Boxes show enlarged notochords, red boundaries show outline of individual notochord cells. In *doc* mutant and 8ng and 4ng doc 2 MO injected embryos there is a lack of notochord differentiation, 'U'-shaped somites and a lack of A-P extension. At 8ng, the trunk completely fails to extend and the notochord can be seen to buckle as it fails to differentiate.

4.5 Expression of *doc* mRNA

Using the constructed doc protein sequence, primers were designed to amplify an 800 base fragment from wild type cDNA. From this, an *insitu* riboprobe was synthesised. This probe was then used to examine the expression of *doc* mRNA in 4-cell, shield stage, tail-bud stage, 6 somite, 14 somite and 24 hpf embryos.

The lack of expression at 4-cell stage demonstrated that this gene is not maternal. Expression analysis also noted a lack of expression at shield stage, tail-bud stage and 6 somite stage. However, there is observable expression specifically within the notochord at 14 somite stage, which is then extinguished by 24 hpf. Demonstrating that expression of *doc* is confined only to the notochord at 14 somite in the stages observed (**Figure 4.4**).

4.6 Notochord Differentiation in doc MO Injected Embryos

Whole mount *insitu* hybridisation of *echidna hedgehog* (*ehh*) was used to examine the differentiation state of the notochord, since the expression of early markers such as *ehh* is maintained in undifferentiated notochords (Stemple et al., 1996).

In 4ng doc 2 MO injected embryos, the expression of *ehh* is maintained specifically within the notochord of 28 hpf embryos. In 28 hpf wild type embryos, the expression of *ehh* is shut down in all but the most posterior tip of the developing notochord. Thus, is appears that MO knock-down of the muliple WD40 domain protein results in a lack of notochord differentiation, similar to that observed in *doc* mutants (**Figure 4.5**).

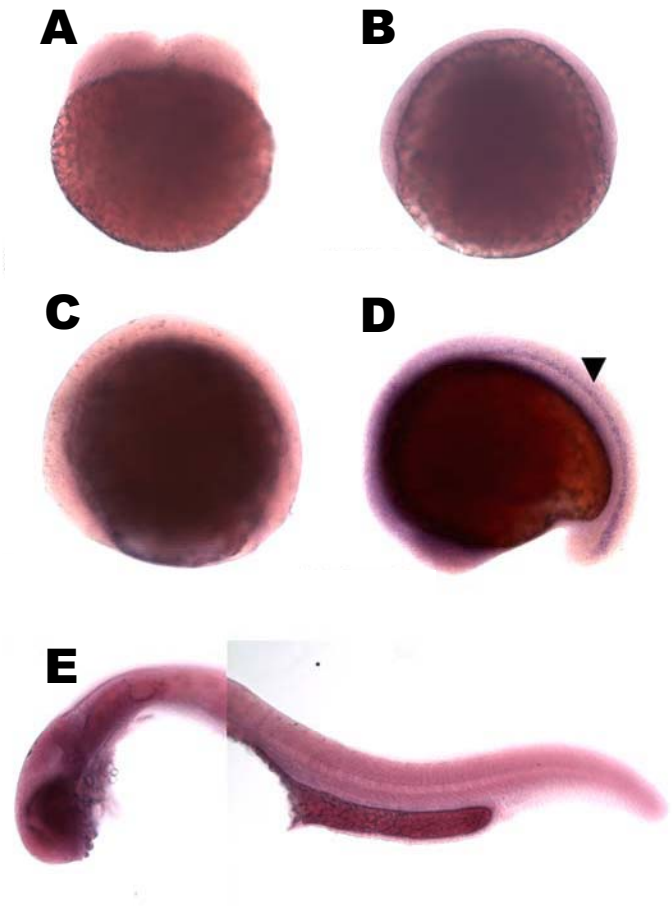


Figure 4.4 Staged expression profile of *doc*.

Lateral views of fixed, staged embryos at (**A**) 2-cell, (**B**) Tail-Bud, (**C**) 6 somite, (**D**) 14 somite and (**E**) 24 hpf. Expression of *doc* is noted only at 14-somite stage specifically within the developing notochord. There is no discernable expression at any other stage.

Figure 4.5 Expression of *ehh* in doc MO injected embryos.

Lateral view of fixed, 24hpf posterior trunk, anterior to the left, dorsal to the top. Expression of *ehh* in 28 hpf wild type embryos (A) is confined to the posterior most developing tip of the notochord. In 4ng doc 2 MO injected embryos (B and C), expression of *ehh* is maintained specifically in the notochord, demonstrating a lack of differentiation.

4.7 Discussion

Positional cloning of *doc* confined the mutation to a 0.5Mb region on linkage group 18. Within this region, several genes were identified from genomic sequence, two of which were deemed likely candidates. The two candidates were Cadherin-13, where cadherins are known interactors to basement membrane, and a novel gene containing multiple WD40 domains. Attempted antisense MO knock-down of Cadherin-13 demonstrated no observable phenotype, though it remains a possibility that the designed MO was unable to fully knock-down the gene product. However, knock-down of *doc* with two separate MOs resulted in embryos bearing significant phenotypic similarity to the *doc* mutants. Such knock-down resulted in 'U' shaped somites, a lack of AP extension and an obvious defect in the differentiation and enlargement of notochord cells. Examination of the this gene demonstrated highly conserved homologues throughout evolution, but was unable to assign function to the protein. Antisense MO knock-down with a secondary MO again phenocopied *doc* and provided stronger evidence that this novel gene encoded *doc*.

To examine the expression profile of *doc* during development an *insitu* riboprobe was generated and used to examine the expression of mRNA in 4-cell, shield, tail-bud, 6 somite, 14 somite and 24 hpf wild type embryos. In the stages examined mRNA was expressed only specifically within the notochord at 14 somite stage. At all other stages expression was absent throughout the entire embryos. So it appears that during the stages of notochord formation and development *doc* is only expressed within the notochord. Though the precise period of notochord expression of *doc* was not defined, the lack of expression in 6 somite and 24 hour embryos suggests that expression is confined to a period between ~11 hpf (6 somite) and 24

hpf. The expression of *doc* specifically within the notochord provides further evidence that it is functioning during notochord development and that mutation of this gene in *doc* embryos is responsible for the specific phenotype of mutant embryos. Since the defining feature of the 'dwarf' mutants is a lack of notochord differentiation, expression of *ehh* within the notochord of doc MO injected embryos was also examined. Expression of *ehh* was noted to be specifically maintained within the notochord of doc 2 MO injected embryos but shut down in wild type embryos of the same stage, thus demonstrating a lack of notochord differentiation in doc 2 MO injected embryos.

The observed cell autonomy of *doc* mutants (Odenthal et al., 1996) and its comparably complete basement membrane has suggested that it may function downstream of BM formation in signalling to the notochord cells and ensuring proper differentiation. Such a role for *doc* fits with the observed expression pattern, where there is an up-regulation prior to the final stages of notochord differentiation and before early markers are extinguished. Further to this, the lack of notochord differentiation, as demonstrated by maintained *ehh* expression, defines a role for doc in proper differentiation of the notochord. However, the novel nature of this gene makes predicting a role or function for this protein in the process of notochord development difficult. The protein demonstrates 14 WD40 domains, and hence may be acting by interacting with other proteins, possibly stabilising BM receptors or may function in organising the cytoskeletal network such that the cells are able to fix sufficiently to the sheath. Defining the precise nature of doc in the process of notochord differentiation is beyond the time scale of this thesis, but how this novel, conserved multiple WD40 domain protein is functioning in the establishment of a fully differentiated notochord remains of great interest.

Though the work presented in these two chapters provides strong evidence that *doc* encodes a novel multiple WD40 domain protein, final proof remains to be provided. Sequencing of the precise mutation from both mutanta cDNA and mutant genomic DNA would provide such evidence. Following that, dCAPS anaylsis can be used to rapidly confirm the presence of the mutation in multiple mutant embryos.

4.8 Summary

- MO knock-down of Cadherin-13 demonstrates no obvious phenotype.
- Antisense MO knock-down with doc1 and doc 1 MO phenocopies *doc* mutants.
- doc encodes a 14 WD40 domain, novel and conserved protein.
- *doc* mRNA is expressed specifically within the notochord at 18 somite stage.
- Antisense MO knock-down of *doc* results in the maintenance of *ehh* in the notochord at 28 hpf.

Chapter Five

Characterisation of *hap, dop* and the COPI Subunits

5 Characterisation of *hap, dop* and the COPI Subunits

In this this chapter I will provide a summary of the process of secretion, paying particular attention to the role and function of coatomer in the secretory network. The processes involved in coatomer vesicle coat formation will be discussed and the composition of the COPI complex will be reviewed. Additionally, current understanding of coatomer function in zebrafish will be briefly covered, leading into work performed as part of this thesis to characterise the *dop* and *hap* mutant loci and to characterise the remaining COPI subunits during zebrafish development.

5.1 Introduction

One of the key features of eukaryotic cells is the presence of an intricate network of endomembranes that function in a system involved with the exchange of macromolecules between cells and their environment. The secretory pathway constitutes a large part of this network, involving the endoplasmic reticulum (ER), Golgi and the trans-Golgi. The secretory network functions to deliver newly synthesised proteins, lipids and carbohydrates to the surface of the cell from the ER, with pre-secretory modification being made as the macromolecules move through the system. Much of the movement involved in this pathway is carried out by the transport of secretory cargo between distinct membrane bound organelles. However, the organelles themselves maintain their characteristic set of resident components. Coated vesicles function in all stages of secretion, acting in transport from the ER to

the Golgi, as in the case of COPII (Barlowe, 1998; Schekman and Orci, 1996), in vesicular trafficking within the endosomal membrane system between the plasma membrane and the trans-Golgi, as in the case of clathrin (Schmid, 1997), in anterograde and retrograde transport within the Golgi (Orci et al., 1997) and in the recycling of component proteins from the Golgi to the ER, as in the case of COPI (Letourneur et al., 1994). The action of these vesicles, specifically the COPI coated vesicles, contribute much to the maintenance of the complement of Golgi resident components.

The current model of the secretory network combines elements of the two classical models of secretion: the vesicular transport model and the cisternal maturation model. The vesicular transport model relies on the premise that the secretory network consists of stable, autonomous organelles and that secretory cargo is transported forward through the secretory network, by COPI and COPII coated vesicles, with 'resident' proteins recycled to their appropriate organelles via COPI mediated transport. The cisternal maturation model holds that the Golgi is a dynamic, steady state system, where secretory cargo is maintained within Golgi compartments and progresses through the Golgi stack as the Golgi compartments mature. In this model, maturation is balanced by a return flow of Golgi resident proteins by COPI vesicles. These two models have been combined to offer a third model, where aspects of both are incorporated. In this third model, large cargos of protein aggregates, for example procollagen, progress through the cisternal maturation model, with smaller cargo molecules being moved rapidly through the secretory system through forward transport in COPI vesicles. However, recent work has suggested that vesicular transport plays only a small part in anterograde transport of secretory cargo (Martinez-Menarguez et al., 2001; Mironov et al., 2001). Thus,

the true model of secretion may be closer to the cisternal maturation model than the vesicular transport model, where the cisternae move in a trans to cis direction as they mature. The transport and modification would thus occur as the cisternae mature, which itself occurs through the retrograde action of COPI vesicles in maintaining the complement of adaptory components at the proper position. This model can be compared to a conveyor belt, where cisternae move forwards, developing as they do so through changes in their compliment of adaptory proteins that proceed to modify the secretory cargo. COPI then functions on the underside of the conveyor belt, to maintain the appropriate type of compartment at the proper trans/cis location through the retrograde transport of resident components (see **Figure 5.1** for overview).

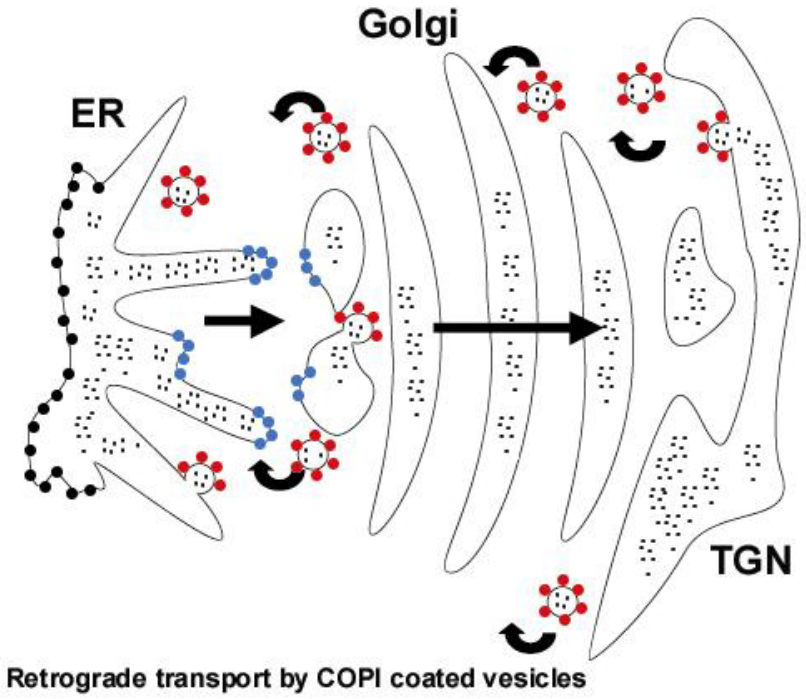


Figure 5.1 Overview of secretion.

Cargo is transported from the ER to the Golgi via COPII action (blue vesicles) and moves through the Golgi network within vesicles via vesicular maturation. Cargo eventually arrive at the trans-Golgi-network (TGN). COPI vesicles (in red) act to maintain Golgi functional identity at a specific trans/cis location through retrograde transport of components, both within the Golgi and from the Golgi to the ER.

COPI is a heptameric complex, made up of the coatomer subunits α, β, β', ε, γ, δ and ζ. Individual coatomer subunits were first identified through the analysis of vesicle coats formed in vitro from Golgi enriched membranes (Orci et al., 1986). In the case of the coatomer γ and ζ subunits, two novel isotypic variants have been identified in mammals (Wegmann et al., 2004) where only one isotype is known for the α, β, β', ε and δ subunits. The COPI subunits form two commonly isolated sub-complexes within the main complex, one consisting of the α, β' and ε subunits, which comprises the B-COPI sub-complex, and the other consisting of the γ, δ., ζ and β subunits, which comprises the F-COPI sub-complex (Fiedler et al., 1996) (**Figure 5.2**). In the case of the F-COPI complex, there is considerable homology to the subunits of the clathrin AP1 and AP2 subunits, where β-COP and γ-COP show homology to the large AP subunits and δ-COP and ζ-COP show homology to the medium and small AP subunits (Boehm and Bonifacino, 2001). More recent work has strengthened the proposed homology between subunits of clathrin and COPI. Structural studies on the γ-COP subunit have demonstrated significant similarity between a carboxyl terminal domain and the carboxyl terminal appendage domains of the α and β subunits of the AP2 unit of clathrin (Hoffman et al., 2003; Watson et al., 2004). This domain was demonstrated to interact with an ARF GTPase Adaptory Protein (ARFGAP) in both yeast and mammalian cells. These data reinforces the idea that both COPI and clathrin act through similar mechanisms in vesicle formation.

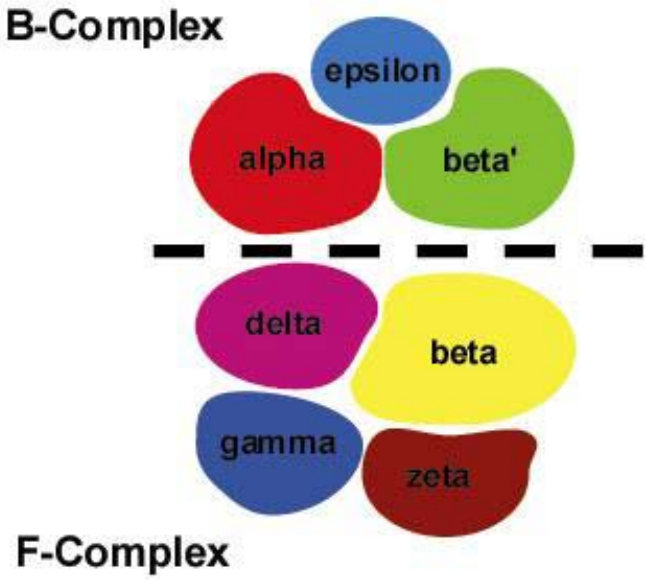


Figure 5.2 COPI complex subunit subcomplex composition.

The COPI complex is formed from two subcomplex's, the B-complex, made up of COPα, COPβ' and COPε, and the F-complex, made up of COPγ, COPβ, COPδ and COPζ. The F-complex bears similarity to the clathrin AP1 and AP2 subunits.

COPI mediated retrograde transport, involved in carrying ER components from post-ER compartments back to the ER is mediated through direct physical interactions between the coat protein and the cargo protein. In the case of proteins such as p24 family members, a C-terminal dilysine (KKxx and KxKxx) motif allows the transport of proteins back to the ER (Cosson et al., 1996; Cosson and Letourneur, 1994; Fiedler et al., 1996). In this instance it is known that the γ subunit recognises these dilysine motifs, thus providing a mechanism for COPI transport of proteins containing these sequences (Harter et al., 1996; Harter and Wieland, 1998). Other proteins, such as BiP, contain a C-terminal Lys-Asp-Glu-Leu KDEL sequence that is recognised by the KDEL receptor and targets proteins for retrograde transport (Munro and Pelham, 1987; Orci et al., 1997). The recent characterisation of the Rer1p protein in yeast, a Golgi membrane protein required for the proper retrieval of a set of ER proteins, as able to interact in vitro with an α/γ coatomer complex and also as a protein that requires proper COPI activity for retrieval, since a lack of COPI activity causes mis-localisation to the vacuole, has demonstrated that other, possibly as yet uncharacterised, mechanisms of COPI cargo targeting may exist (Sato et al., 2001). COPI is also thought to act, somewhat indirectly, in localising proteins to the Golgi through retrieval of Golgi components from the vacuole to the Golgi. This has again been shown to be mediated by COPI binding to lysine rich, though not the conventional dilysine, C-terminal domains (Abe et al., 2004). Finally, some COPI coated vesicles have been observed carrying anterograde bound cargo, since a distinct population of vesicles was noted that carried cargo including proinsulin and VSV G protein (Orci et al., 1997). Thus, an as yet uncharacterised domain may act in directing cargo in an anterograde direction, in the manner that the KDEL sequence and dilysine motifs can direct cargo in a retrograde COPI pathway.

COPI dependent vesicular budding relies on the GTP dependent recruitment of GDP-bound ARF1 and COPI to Golgi membranes (Donaldson et al., 1992; Palmer et al., 1993; Rothman, 1994). The GTP loading of ARF1 is catalysed by GEFs, where the ARF1 interacting GEFs in yeast have been demonstrated to share a Sec7 domain (Peyroche et al., 1999). ARF1-GDP interacts specifically with the Golgi before nucleotide exchange (Gommel et al., 2001). In this context p23, a type I transmembrane protein, belonging to the p24 family, is known to play a key role in COPI coat assembly and has been identified as an ARF1-GDP receptor (Bremser et al., 1999; Gommel et al., 2001; Sohn et al., 1996). This p23 interacting ability has been localised to the c-terminal 22 residues of ARF1. However, ARF1 nucleotide exchange can take place in the presence of liposome's lacking any protein, and hence binding to p23 is not a requirement for nucleotide exchange (Beraud-Dufour et al., 1999). Though in a biological context p23 may act to direct ARF1 to desired sites and thus to its GEF. Interestingly, ARF hydrolysis of GTP is itself affected by p24 family members, so that ARF1 is likely undergoing continual cycles between the GDP and GTP bound states. Since only the GTP bound form of ARF1 interacts with the membrane, through the exposure of a myristoyl group (Goldberg, 1998), p23 may also function to maintain the concentration of ARF1-GDP at the Golgi membrane. Nucleotide exchange instigates release of ARF1-GTP from p23 (Gommel et al., 2001), generating two binding sites for COPI at the membrane; on ARF1-GTP (Zhao et al., 1997; Zhao et al., 1999), and on p23 (Dominguez et al., 1998; Sohn et al., 1996). However, the recruitment of COPI depends entirely on the proper activation and membrane localisation of ARF1 (Donaldson et al., 1992; Palmer et al., 1993). Following this recruitment, coatomer and ARF1-GTP polymerise to from a defined macromolecular structure that is likely to limit the size of forming vesicles (Bremser

et al., 1999; Reinhard et al., 2003). The rate of GTP hydrolysis and release of ARF1 from membranes has been suggested to act as a timer, triggering release of coat protein so that vesicles may fuse with target membranes. The rate of hydrolysis of GTP by ARF1 depends on the interaction with ARFGAP and COPI (Goldberg, 1999). However, recently the carboxy terminal FFxxRRxx sequence of the p24 protein hp24a, which binds to COPI, has been shown to reduce the ability of COPI to stimulate ARFGAP, though the FFxxKKxx sequence, in proteins that also bind COPI do not (Goldberg, 2000). This may represent 'preferred' cargo, which would fit with a model where COPI vesicles are continually forming on membranes and that only those that capture the 'preferred' cargo are stabilised, through inhibition of COPI stimulation ARFGAP, and are thus able to complete coat formation.

ARF GTPases are activated by GEFs of the Sec7 family, which promote the exchange of GDP for GTP (Peyroche et al., 1996). This oscillation between the GDP and GTP bound states switches ARF between the 'non membrane binding' and 'membrane binding' state (Amor et al., 1994; Goldberg, 1998). ARF1 activity is vital for coatomer coat formation and its activity can be inhibited through the activity of the fungal metabolite brefeldin A (BFA) (Donaldson et al., 1992; Helms and Rothman, 1992). Treatment of cell with BFA causes a redistribution of COPI components to the cytoplasm, a loss of COPI vesicle formation, a redistribution of Golgi components to the ER and a breakdown of Golgi structure (Donaldson et al., 1990; Lippincott-Schwartz et al., 1989; Orci et al., 1991). It has been demonstrated that BFA inhibits the action of GEFs for ARF1, blocking ARF1 activation and therefore the proper assembly of COPI vesicles (Donaldson et al., 1992; Helms and Rothman, 1992). More recent work has demonstrated that this inhibition occurs

through the stabilisation of the ARF1-GDP-Sec7 complex, rather that through the inhibition of interactions between Sec7 GEFs and ARF1 (Peyroche et al., 1999).

Loss of coatomer function in yeast, mammalian and zebrafish cells is lethal (Coutinho et al., 2004; Gerich et al., 1995; Guo et al., 1994). Thus, coatomer can be assumed to have a 'house-keeping' function, being necessary in all cells to maintain the secretory networks. However, it has been demonstrated specifically that loss of some coatomer subunits in zebrafish result in specific developmental phenotypes before widespread lethality is apparent ((Coutinho et al., 2004), this thesis). Moreover, COPα has been demonstrated to be expressed in a manner suggesting that demand for and activity of COPI acts in some way to regulate the expression of *COPα* and thus control the pool of available coatomer activity. In this section, I will characterise the coatomer subunits that are responsible for two zebrafish mutants, *dop* and *hap*. In addition, I will examine the expression profile of the remaining COPI subunits, both during normal development and under conditions where COPI activity is lost, to examine the possible regulation of COPI subunit expression by available coatomer function.

5.2 Identifying Mutations in *hap*^{tm285b} and *dop*^{m341}

5.2.1 Radiation Hybrid Mapping of COPβ

Using publicly available sequence EST and genomic sequence data for *COP*β, two primer sets were designed to RH map the loci. Using the LN54 zebrafish radiation hybrid panel (Hukriede et al., 1999), *COP*β was mapped to within 12.90

centi-Rays (cR) of the marker z20853 on LG7. Previous mapping efforts had successfully linked *hap* to the same LG.

5.2.2 Radiation Hybrid mapping of COPβ'

As for *COP*β, publicly available sequence EST and genomic sequence data for *COP*β' was used to design two primer sets to RH map the loci. Using the LN54 zebrafish radiation hybrid panel (Hukriede et al., 1999), *COP*β' was mapped to within 7.47 cR of *elrA* on LG2. As with *hap*, previous mapping efforts had successfully linked *dop* to the same LG.

5.2.3 Cloning the Full Length COPβ Gene

Publicly available EST and genomic data was compared to the mouse COPβ to generate a scaffold of known sequence. This was then used to design primers to cover the gaps in available sequence from cDNA, 3' and 5' RACE products. Sequencing of these products allowed the reconstruction of a full length cDNA of 3,239 bases. The generated cDNA sequence predicted a protein of 953aa that demonstrated 93% identity to both the human and mouse *COP*β genes.

5.2.4 Identification of the Mutation in *hap*^{tm285b}

Using the full length cDNA sequence, primers were designed to generate overlapping COPβ cDNA fragments from *hap*^{tm285b} mutant embryos. Sequencing of these fragments identified a non-sense mutation in the codon encoding residue E_{499}, where a G to T substitution produces an early termination. Confirmation of this mutation was provided through re-sequencing of the loci containing the mutation from genomic DNA obtained from *hap*^{tm285b} mutant embryos. Final confirmation of the identified mutation in *hap*^{tm285b} mutants was provided through dCAPS analysis, whereby the identified mutation was shown to be present in all six mutant embryos but absent in one of the six wild type embryos, with the remaining five demonstrating heterozygosity.

5.2.5 Cloning the Full Length COPβ' Gene

Publicly available EST and genomic data was compared to the mouse COPβ' to generate a scaffold of known sequence. This was then used to design primers to cover the gaps in available sequence from cDNA, 3' and 5' RACE products. Sequencing of these products allowed the reconstruction of a full length cDNA of 3,170 bases. The generated cDNA sequence predicted a protein of 934aa that demonstrated 86% and 87% identity to the human and mouse *COP*β' genes respectively.

5.2.6 Identification of the Mutation in *dop*^{m341}

Using the full length cDNA sequence, primers were designed to generate overlapping COPβ' cDNA fragments from *dop*^{m341} mutant embryos. Sequencing of these fragments identified a non-sense mutation in the codon encoding residue Y_{761}, where a T to A substitution produces an early termination. Confirmation of this mutation was provided through re-sequencing of the loci containing the mutation from genomic DNA obtained from *dop*^{m341} mutant embryos. Final confirmation of the identified mutation in *dop*^{m341} mutants was provided through dCAPS analysis, whereby the identified mutation was shown to be present in all six mutant embryos tested but absent in two of the six wild type embryos, with the remaining four demonstrating heterozygosity.

5.3 Expression of COPβ and COPβ'

5.3.1 Expression of COPβ mRNA

Using the generated full length cDNA sequence, primers were designed to amplify a 997bp fragment from wild type cDNA. From this, an *insitu* riboprobe for *COP*β was synthesised. This probe was then used to examine the expression of *COP*β in staged wild type embryos; at approximately 32 cell stage, shield stage, tail-bud stage, 5 somite stage, 14 somite stage, 24 hpf and in the three coatomer mutants; *sny, hap* and *dop* at 28 hpf.

*COP*β is first observed at 32 cell stage, demonstrating that it is maternally expressed. The expression continues through to shield stage, when expression is

ubiquitous. By tail-bud stage, the general ubiquitous expression is lower, though still present and there is a noticeable up-regulation specifically within the chordamesoderm. This chordamesoderm/notochord localised up-regulation continues through to 14 somites. Expression above the background levels are also detected in the developing brain during somitogenesis. However, by 24 hpf expression is returned to low levels of ubiquitous expression throughout the embryo with the exception of the most posterior tip of the developing notochord, where notochord cells are not yet fully differentiated (**Figure 5.3**).

The comparison of *COP*β expression in both mutant and wild type sibling *sny*, *hap* and *dop* mutants demonstrated that the chordamesoderm/notochord expression of *COP*β is maintained in 28 hpf *sny*, *hap* and *dop* embryos whereas in 28 hpf sibling wild type embryos, the chordamesoderm specific up-regulation is extinguished. The background levels of *COP*β remain unaffected in *sny*, *hap* and *dop* mutants (**Figure 5.4**).

5.3.2 Expression of *COP*β' mRNA

Using the generated full length cDNA sequence, primers were designed to amplify a 929bp fragment from wild type cDNA. From this, an *insitu* riboprobe for *COP*β' was synthesised. This probe was then used to examine the expression of *COP*β' in staged wild type embryos; at approximately 32 cell stage, shield stage, tail-bud stage, 5 somite stage, 14 somite stage, 24 hpf and in the three coatomer mutants; *sny*, *hap* and *dop* at 28 hpf.

As with *COP*β, *COP*β' is maternally expressed, with expression continuing through to shield stage at which point expression is ubiquitous. By tail-bud, the ubiquitous level of expression is lower, though still present and expression within the chordamesoderm is specifically up-regulated. This chordamesoderm/notochord localised up-regulation continues through to 14 somites. Elevated levels of expression are also detected in the developing brain during somitogenesis. By 24 hpf expression is returned to low levels of ubiquitous expression throughout the embryo with the exception of the most posterior tip of the developing notochord (**Figure 5.5**).

Comparison of *COP*β' expression in both mutant and wild type sibling *sny*, *hap* and *dop* mutants demonstrated that, as observed with *COP*β the chordamesoderm/notochord expression of *COP*β' is maintained in 28 hpf *sny*, *hap* and *dop* embryos whereas in 28 hpf sibling wild type embryos the chordamesoderm specific up-regulation is extinguished. Background levels of *COP*β' remain unaffected in *sny*, *hap* and *dop* mutants (**Figure 5.6**).

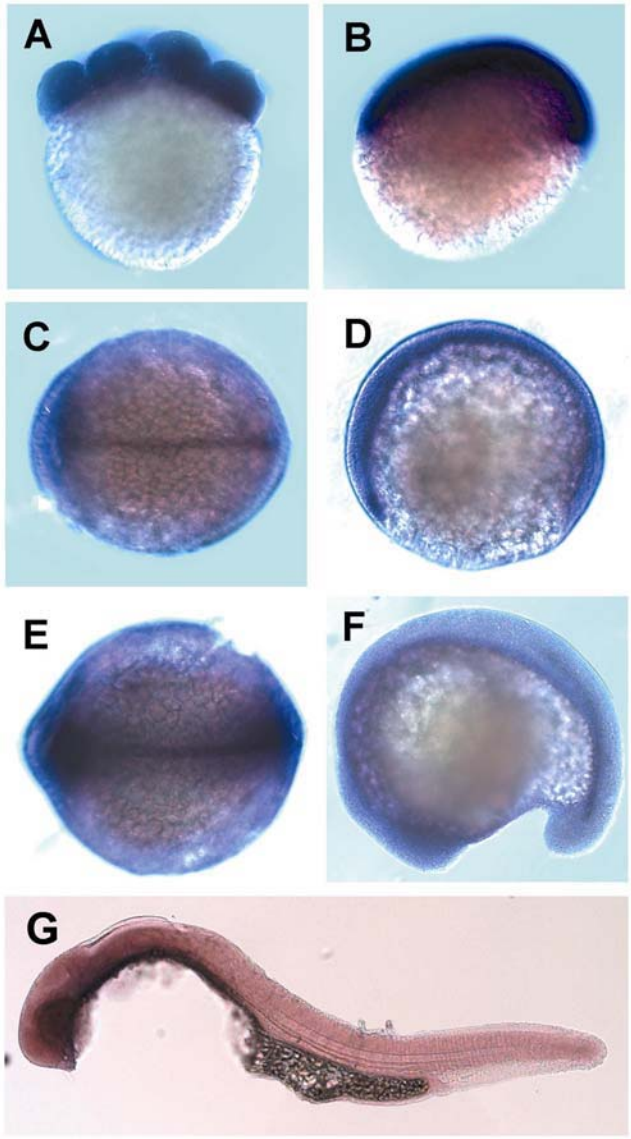


Figure 5.3 Staged expression profile of *COP*β.

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view, (**F**); and lateral view, anterior to the left, of 14 somite and (**G**) 24 hpf. (**A**) High level of expression at 32 cell stage demonstrates that *COP*β is provided maternally. At shield stage (**B**) expression is ubiquitous and at relatively high levels. By tailbud (**C** and **D**) stage, the ubiquitous expression is lower with the exception of specific up-regulation within the chordamesoderm. This is maintained through 5 somite (**E**) and 14 somite stages (**F**), with noticeably high expression in the developing brain at these stages. By 24 hpf (**G**) notochord specific expression has been extinguished and up-regulation is confined to the developing brain.

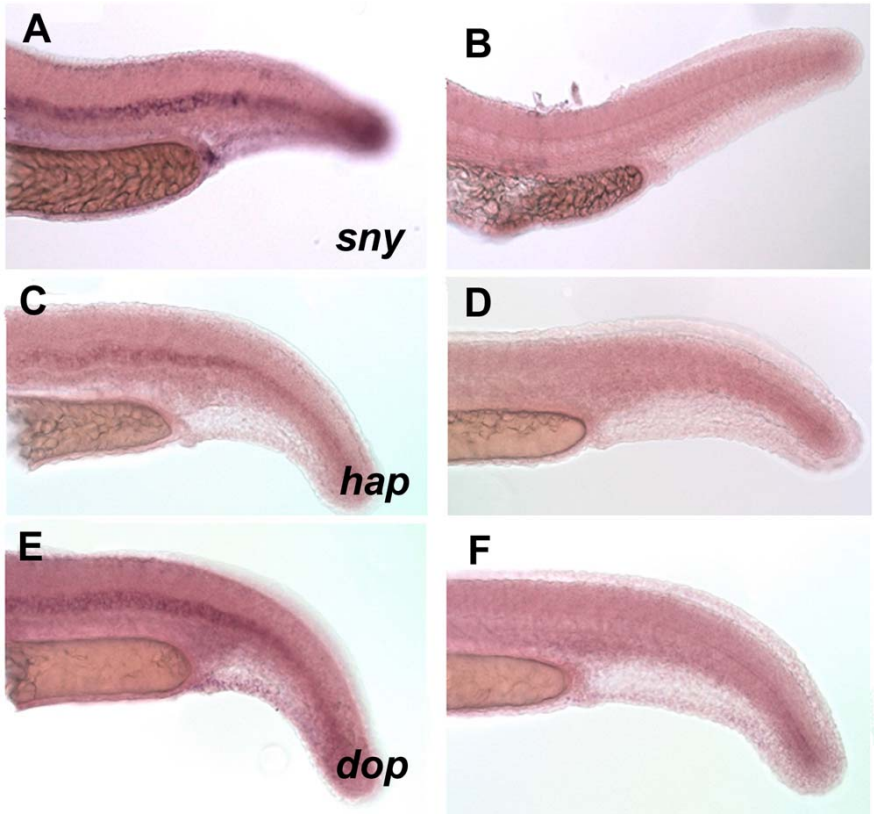


Figure 5.4 Expression profile of *COP*β in COPI mutants and wild type embryos at 28 hpf.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top. Tails taken from mutant embryos (**A**, **C**, **E**) and wildtype siblings (**B**, **D**, **F**).
At 28 hpf, the Expression of *COP*β is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*β is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

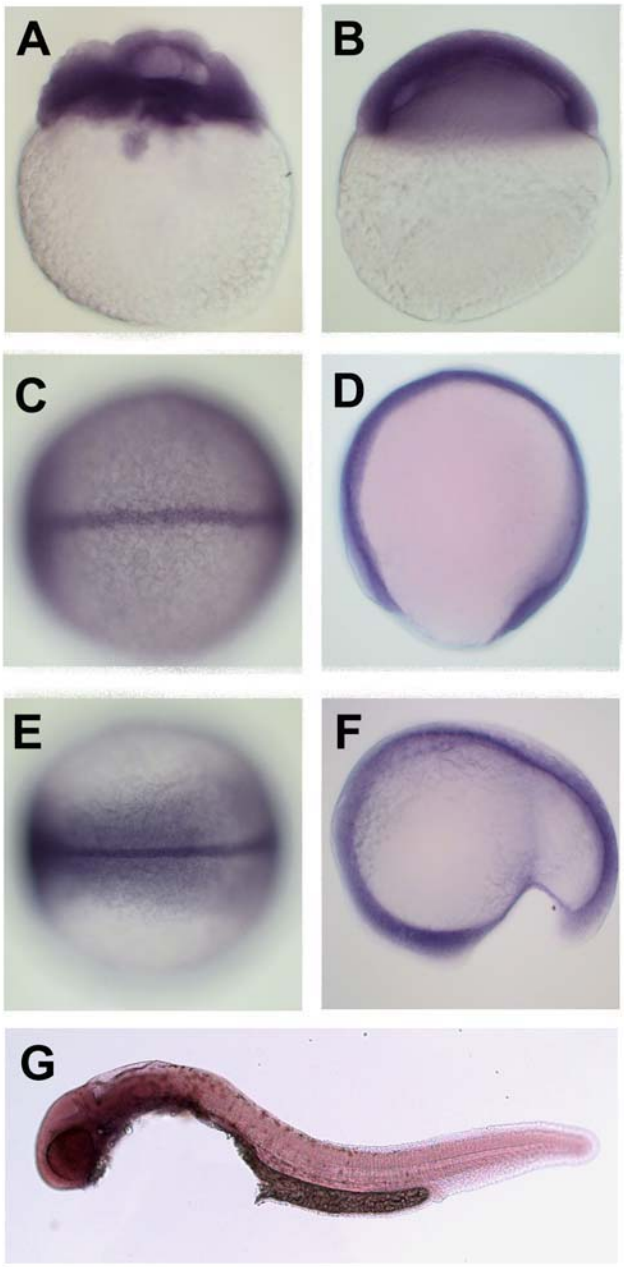


Figure 5.5 Staged expression profile of *COPβ*'.

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view, (**F**); and lateral view, anterior to the left, of 14 somite and (**G**) 24 hpf. (**A**) High level of expression at 32 cell stage demonstrates that *COP*β' is provided maternally. At shield stage (**B**) expression is ubiquitous and at relatively high levels. By tailbud (**C** and **D**) stage, the ubiquitous expression is lower with the exception of specific up-regulation within the chordamesoderm. This is maintained through 5 somite (**E**) and 14 somite stages (**F**), with noticeably high expression in the developing brain at these stages. By 24 hpf (**G**) notochord specific expression has been extinguished and up-regulation is confined to the developing brain.

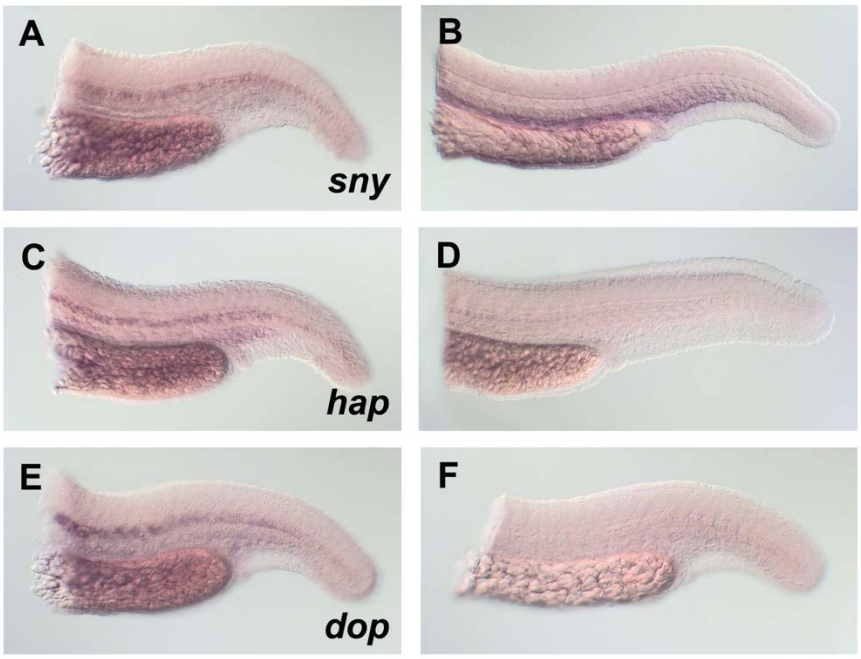


Figure 5.6 Expression profile of *COPβ'* in mutant and wild type embryos at 28 hpf.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top. Tails taken from mutant embryos (**A**, **C**, **E**) and wildtype siblings (**B**, **D**, **F**).
At 28 hpf, the Expression of *COP*β' is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*β' is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

5.4 Identification of the zebrafish COPI subunits

Publicly available zebrafish EST and genomic data was compared to the mouse COPε, COPδ, COPγ, COPγ2, COPζ and COPζ2 genes to generate scaffolds of known sequence. Compiled EST data predicted cDNAs of 1258, 1818, 3005, 1034 and 915 bases for COPε, COPδ, COPγ2, COPζ1 and COPζ2 respectively. A homologue for zebrafish COPγ1 has yet to be found and exhaustive EST searches have returned no sequence, however, the incomplete nature of the genome and the presence of two COPγ genes in many other higher organisms suggests that a secondary COPγ homologue has yet to be identified. The generated cDNA sequences for COPε, COPδ, COPγ2, COPζ1 and COPζ2 predicted proteins of 481, 509, 873, 198 and 189 amino acids respectively.

5.5 Expression of COPI subunit mRNA

Using the generated sequence for *COPε*, *COPδ*, *COPγ2*, *COPζ* and *COPζ2* primers were designed to amplify a 722bp, 932bp, 790bp, 864bp, and 742bp fragments respectively, from wild type cDNA. From these fragments, *insitu* riboprobes for the *COPI* subunits were synthesised. These probes were then used to examine the expression of the *COPI* subunits in staged wild type embryos; at approximately 32 cell stage, shield stage, tail-bud stage, 5 somite stage, 14 somite stage, 24 hpf and in the three coatomer mutants; *sny*, *hap* and *dop* at 28 hpf.

5.5.1 Expression of *COPε*, *COPδ*, *COPγ2* and *COPζ2* mRNA

Similar to the expression of *COP*β and *COP*β',the COPI subunits ε, δ, γ2 and ζ2 are all maternally expressed. Ubiquitous expression continues through to shield stage and by tail-bud stage, the ubiquitous level of expression is lower, though still present. At this stage, expression within chordamesoderm cells is specifically up-regulated, as observed with *COP*β and *COP*β'. This specific chordamesoderm/notochord up-regulation continues through to 14 somites. Expression of all subunits is elevated within the developing brain during somitogenesis. As with *COP*β and *COP*β', expression of these COPI subunits is returned to low levels of ubiquitous expression throughout the embryo, with the exception of the most posterior tip of the developing notochord, by 28 hpf (**Figure 5.7 through to 5.10**).

Expression of *COPε*, *COPδ*, *COPγ*2 and *COPζ*2 in both mutant and wild type sibling *sny*, *hap* and *dop* mutants demonstrated that, as observed with *COP*β and *COP*β', the chordamesoderm/notochord expression of these COPI subunits is maintained in 28 hpf *sny*, *hap* and *dop* embryos whereas in 28 hpf sibling wild type embryos the chordamesoderm specific up-regulation is extinguished. Background levels of these COPI subunits remain unaffected in *sny*, *hap* and *dop* mutants (**Figure 5.11 through to 5.14**).

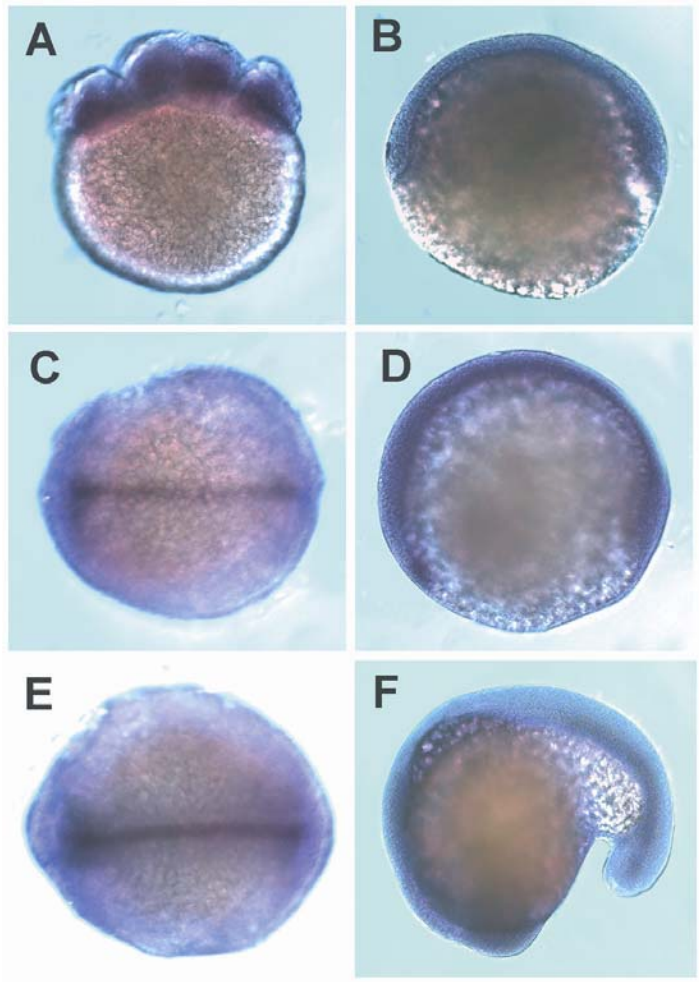


Figure 5.7 Staged expression profile of *COPε*.

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view and (**F**) 14 somite lateral view, anterior to the left. (**A**) High level of expression at 32 cell stage demonstrates that *COPε* is provided maternally. At shield stage (**B**) expression is ubiquitous and at relatively high levels. By tailbud (**C** and **D**) stage, the ubiquitous expression is lower with the exception of specific up-regulation within the chordamesoderm. This is maintained through 5 somite (**E**) and 14 somite stages (**F**), with noticeably high expression in the developing brain at these stages.

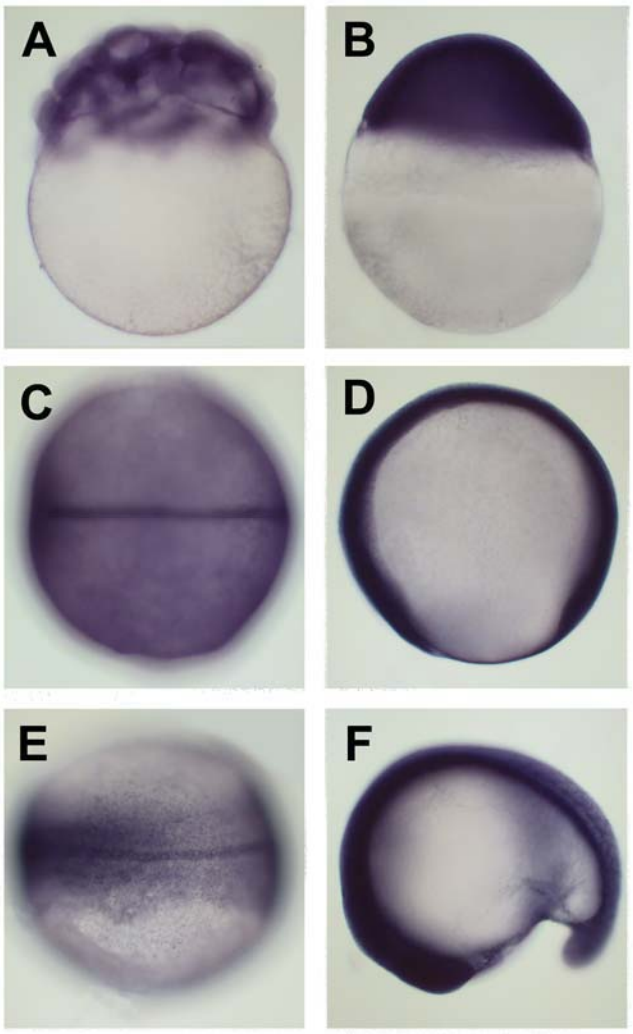


Figure 5.8 Staged expression profile of *COP*δ

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view and (**F**) 14 somite lateral view, anterior to the left. (**A**) High level of expression at 32 cell stage demonstrates that *COPδ* is provided maternally. At shield stage (**B**) expression is ubiquitous and at relatively high levels. By tailbud (**C** and **D**) stage, the ubiquitous expression is lower with the exception of specific up-regulation within the chordamesoderm. This is maintained through 5 somite (**E**) and 14 somite stages (**F**), with noticeably high expression in the developing brain at these stages.

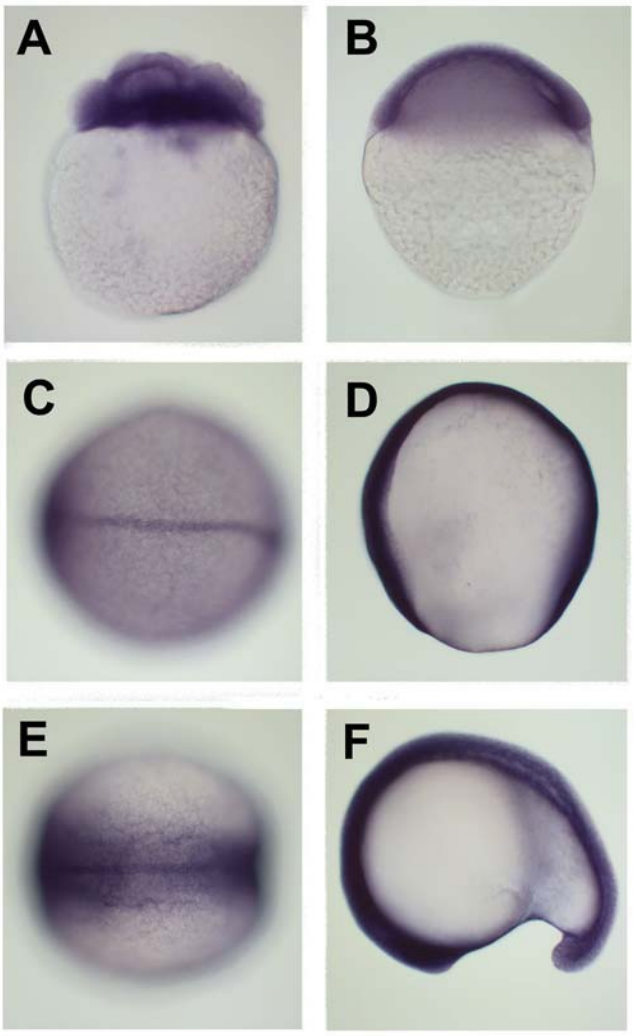


Figure 5.9 Staged expression profile of *COPγ2*

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view and (**F**) 14 somite lateral view, anterior to the left. (**A**) High level of expression at 32 cell stage demonstrates that *COPγ2* is provided maternally. At shield stage (**B**) expression is ubiquitous and at relatively high levels. By tailbud (**C** and **D**) stage, the ubiquitous expression is lower with the exception of specific up-regulation within the chordamesoderm. This is maintained through 5 somite (**E**) and 14 somite stages (**F**), with noticeably high expression in the developing brain at these stages.

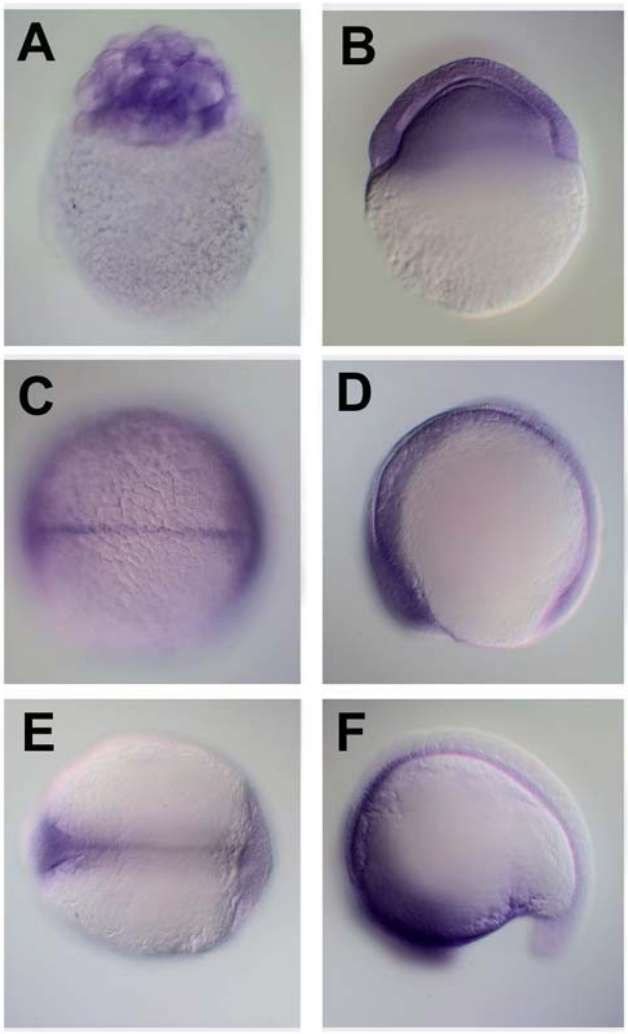


Figure 5.10 Staged expression profile of *COPζ2*

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view and (**F**) 14 somite lateral view, anterior to the left. (**A**) High level of expression at 32 cell stage demonstrates that *COPζ2* is provided maternally. At shield stage (**B**) expression is ubiquitous and at relatively high levels. By tailbud (**C** and **D**) stage, the ubiquitous expression is lower with the exception of specific up-regulation within the chordamesoderm. This is maintained through 5 somite (**E**) and 14 somite stages (**F**), with noticeably high expression in the developing brain at these stages.

Figure 5.11 Expression profile of *COP*ε in mutant and wild type embryos at 28 hpf.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top. Tails taken from mutant embryos (**A**, **C**, **E**) and wildtype siblings (**B**, **D**, **F**).
At 28 hpf, the Expression of *COP*ε is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*ε is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

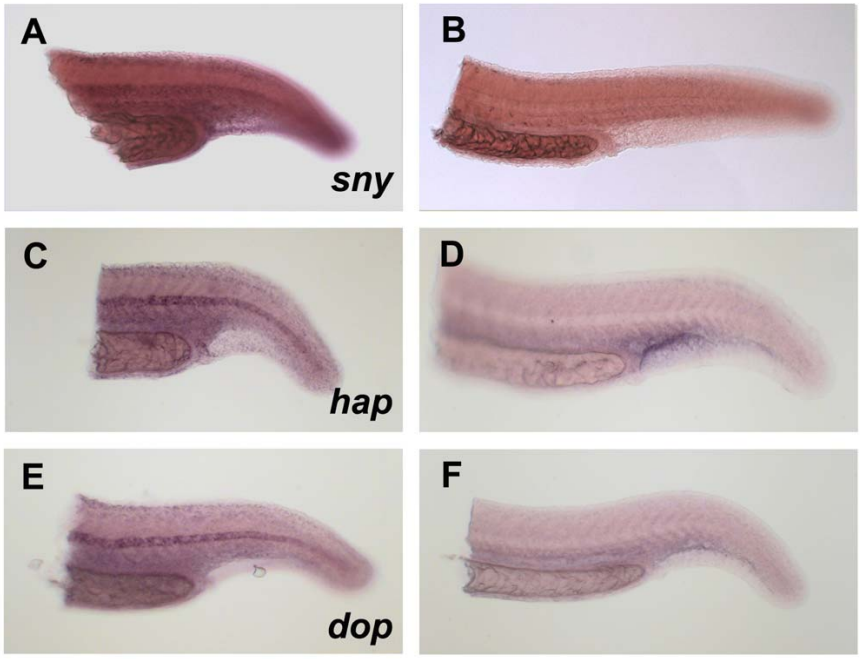


Figure 5.12 Expression profile of *COP*δ in mutant and wild type embryos at 28 hpf.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top. Tails taken from mutant embryos (**A**, **C**, **E**) and wildtype siblings (**B**, **D**, **F**).
At 28 hpf, the Expression of *COP*δ is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*δ is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

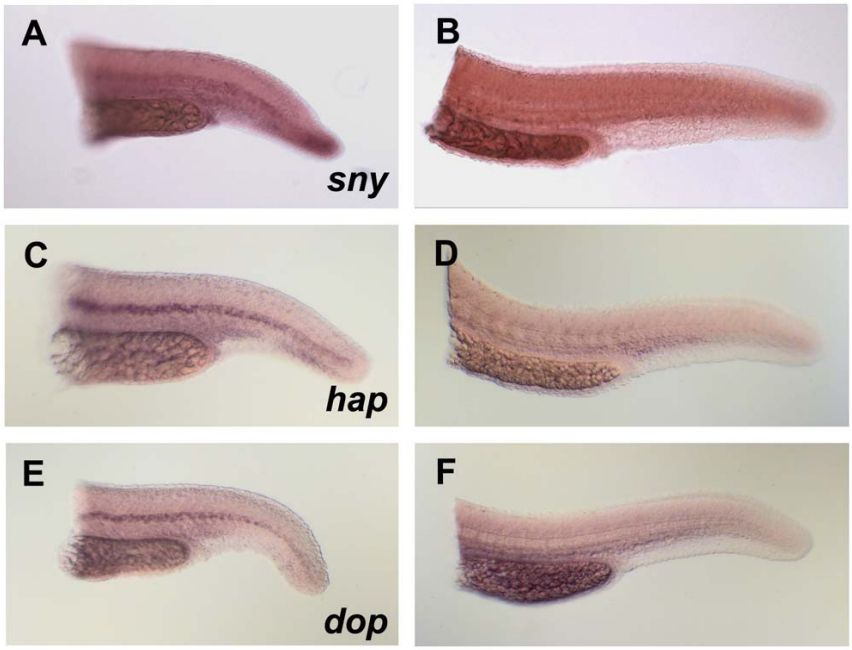


Figure 5.13 Expression profile of *COP*γ2 in mutant and wild type embryos at 28 hpf.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top. Tails taken from mutant embryos (**A**, **C**, **E**) and wildtype siblings (**B**, **D**, **F**).
At 28 hpf, the Expression of *COP*γ2 is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*γ2 is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

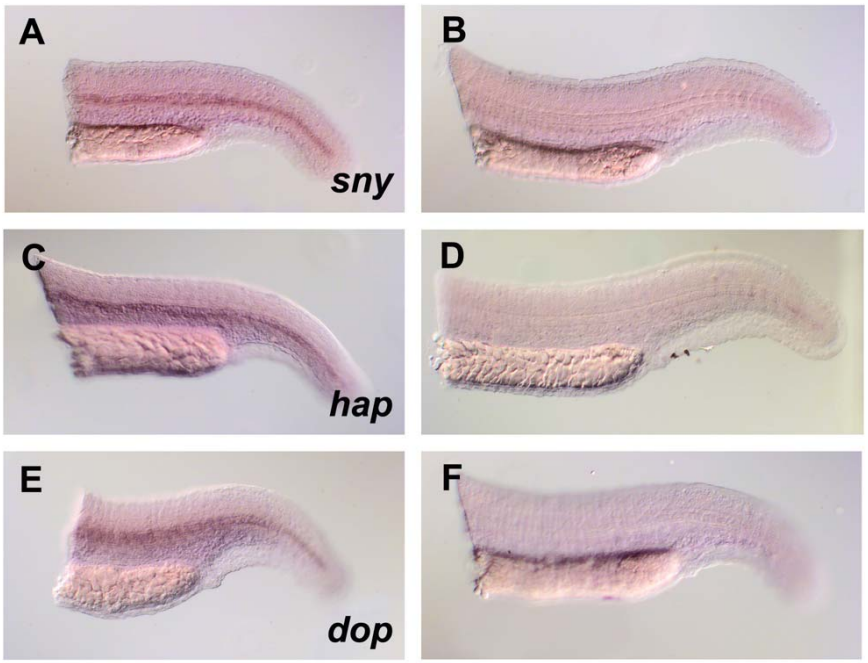


Figure 5.14 Expression profile of *COP*ζ2 in mutant and wild type embryos at 28 hpf.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top. Tails taken from mutant embryos (**A**, **C**, **E**) and wildtype siblings (**B**, **D**, **F**).
At 28 hpf, the Expression of *COP*ζ2 is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*ζ2 is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

5.5.2 Expression of *COPζ* mRNA

Expression of the *COPζ* subunit differs from that of the other coatomer subunits. *COPζ* are first observed at the 32 cell stage, indicating maternal expression. By shield stage, the expression of *COPζ* is considerably lower than that observed for other subunits, though there are low levels of expression throughout the embryo. This low level ubiquitous expression continues through tail bud stage and throughout somitogenesis, with no up-regulation in the chordamesoderm/notochord. However, *COPζ* does show high levels of expression in the developing brain (**Figure 5.15**).

The comparison of expression of *COPζ* in both mutant and wild type sibling *sny*, *hap* and *dop* mutants demonstrated that there is no notochord expression of either subunit in either mutant embryos or their wild type siblings at 28 hpf (**Figure 5.16**).

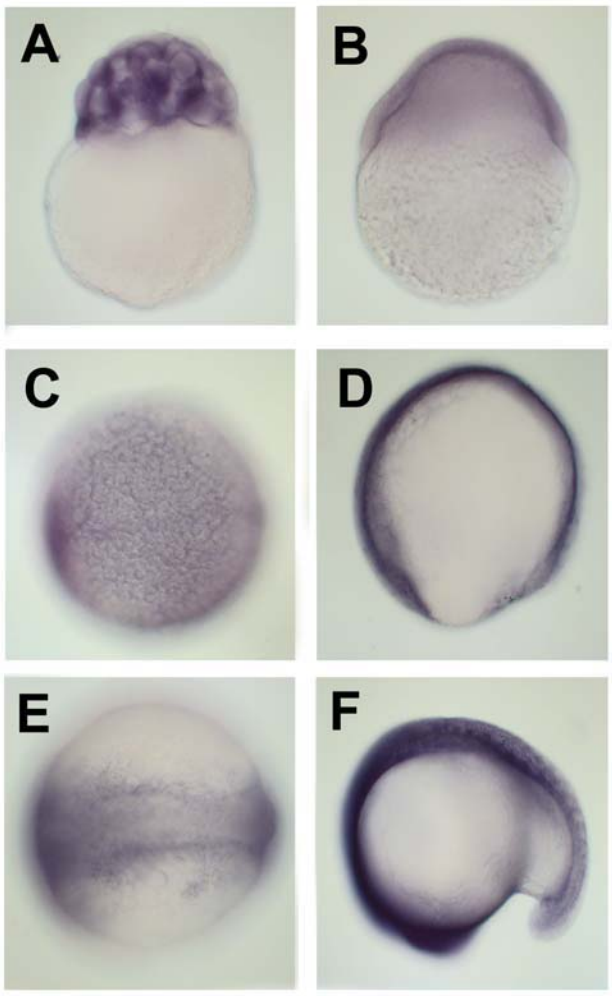


Figure 5.15 Staged expression profile of *COPζ*

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view and (**F**) 14 somite lateral view, anterior to the left. (**A**) High level of expression at 32 cell stage demonstrates that *COPζ* is provided maternally. At shield stage (**B**) expression is ubiquitous and at relatively high levels. By tailbud (**C** and **D**) stage, the ubiquitous expression is lower and there is a lack of the specific up-regulation within the chordamesoderm observed with other COPI subunits. This is continued through 5 somite (**E**) and 14 somite stages (**F**), with noticeably high expression in the developing brain at these stages.

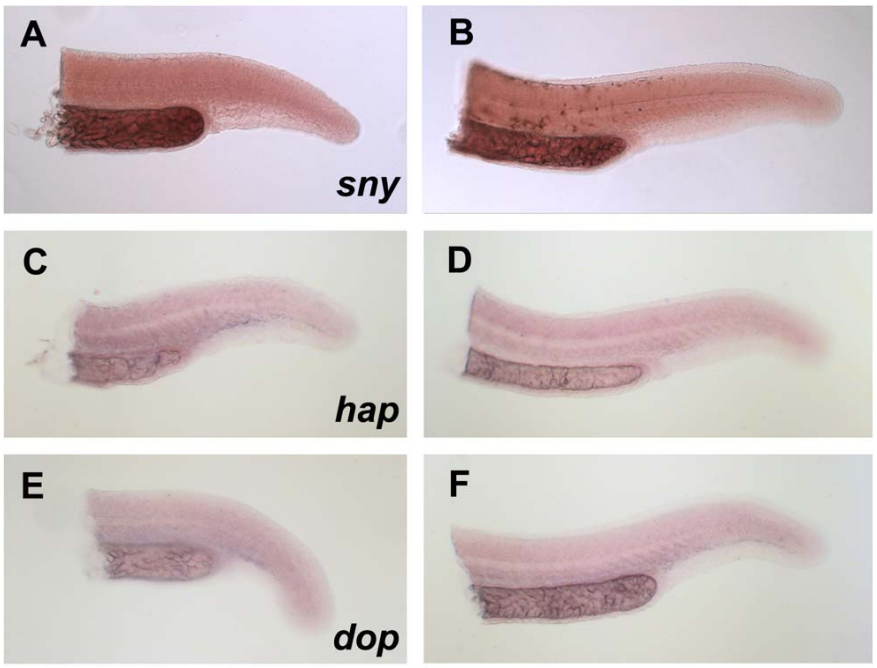


Figure 5.16 Expression profile of *COP*ζ in mutant and wild type embryos at 28 hpf.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top. Tails taken from mutant embryos (**A**, **C**, **E**) and wildtype siblings (**B**, **D**, **F**).
At 28 hpf, the Expression of *COP*ζ is not maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *COP*ζ is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

5.6 Discussion

The *hap* and *dop* loci were suggested to encode subunits of the COPI complex based on the identification of *COPα* as the gene responsible for *sny* (Coutinho, 2001). This work suggested that the *hap* and *dop* loci encoded *COP*β and *COP*β', demonstrating that injection of antisense MO's against *COP*β and *COP*β' could indeed phenocopy *hap* and *dop*.

As part of this thesis, I cloned the full length cDNA for both *COP*β and *COP*β'. The full length cDNAs are 3239 bases and 3170 bases long, encoding proteins of 953aa and 934aa respectively. The zebrafish COPβ shows 93% identity to both the human and mouse COPβ, whereas zebrafish COPβ' shows 86% and 87% identity to the human and mouse homologues of COPβ' respectively. Using these full length cDNA sequence, *COP*β and *COP*β' cDNA was sequenced from *hap* and *dop* mutant embryos, identifying a G to T substitution at residue E_{499} in *hap*^{tm285b} embryos and a T to A substitution in *dop*^{m341} embryos at residue Y_{761}. Both mutations result in premature termination of the protein. Hence, the mutants *hap* and *dop* lack a functional COPI complex and the three notochord mutants *sny, hap* and *dop*, which all present almost indistinguishable phenotypes, all lack vital subunits of the essential coatomer complex. These mutants are all therefore deficient in COPI function.

Expression profiling of *COP*β and *COP*β' demonstrated that, like *COPα*, mRNA is provided maternally. During development, expression is ubiquitous with high levels of specific up-regulation occurring within the developing chordamesoderm and notochord. Once the notochord has differentiated this specific

expression is shut down, similar to other early notochord markers, such as *ehh*. Examination of the expression of *COP*β and *COP*β' within mutants and wild type siblings at 28 hpf demonstrated a maintenance of COPβ and COPβ' mRNA within the undifferentiated notochord of mutant embryos. This is, again, similar to the observation that other early notochord markers are maintained beyond their normal temporal expression domain in mutants demonstrating a failure of notochord differentiation.

In an effort to fully characterise the coatomer complex, I cloned the remaining subunits; *COPε*, *COPδ*, *COPγ2*, *COPζ* and *COPζ2* and used the scaffolds to generate *insitu* probes to examine their expression profile. Such an examination revealed that expression of *COPε*, *COPδ*, *COPγ2* and *COPζ2* is, like *COPα*, *COP*β and *COP*β', maternal and ubiquitous throughout early development, with a specific up-regulation within the chordamesoderm/notochord during development that is shut down by 24 hpf. These subunits were also demonstrated to be maintained within the notochord of the COPI mutants *sny, hap* and *dop* at 28 hpf though not in the differentiated notochord of wild type siblings. Hence, a complete set of seven COPI subunits, which are thus capable of forming a functional coatomer complex, are up-regulated specifically within the developing notochord and maintained beyond their normal temporal expression domain in the mutants *sny, hap* and *dop*, which fail to form a fully differentiated notochord. This maintenance of COPI subunit expression within mutants lacking COPI function suggests the possibility of a regulatory feedback mechanism where COPI functions both in the secretory network and in repressing expression of the coatomer subunits α, β, β', ε, δ, γ2 and ζ2. Though it remains a possibility that COPI subunits are maintained within the notochord of the COPI mutants merely due to a lack of notochord differentiation, as is observed with

notochord markers such as *ehh* (Parsons et al., 2002b; Stemple et al., 1996), rather than the specific loss of coatomer function.

Finally, the expression of *COPζ* differed from the observed expression of the other coatomer subunits. This subunit shows maternal expression, and is expressed ubiquitously at low levels at later stages and within the developing brain during somitogenesis, as observed for the other COPI subunits. However, *COPζ* is not up-regulated within the chordamesoderm and notochord during development and is also not present with the notochord of the COPI mutants at 28 hpf. Hence, only those subunits that are up-regulated with the chordamesoderm during early notochord development are maintained within the undifferentiated notochord of COPI mutants at 28 hpf.

Interestingly, insertional mutagenesis screening in zebrafish has generated a mutant for COPζ1 (Golling et al., 2002). These mutants were reported to show degeneration of the eyed after 4 days development, with no reports of notochord or melanophore defects, which befits its specific expression within the developing head. It may be that other COPI subunits, which are also up-regulated within the head, are also required for proper eye development. However, loss of COPα, COPβ or COPβ' in *sny, hap* or *dop* embryos results in loss of COPI function throught the embryo where loss of COPζ1 does not, since COPζ2 can function in its place, and thus eye defects are not observed in these mutants due to early lethality. The specific expression profiles of *COPζ1* and *COPζ2* combined with the specific eye defect in COPζ1 mutants (Golling et al., 2002) suggests that these two subunits may function in distinct developmental processes.

5.7 summary

- The zebrafish mutant *hap*^{tm285b} has a G→T substitution at residue E_{499}, which results in early termination.
- The zebrafish mutant *dop*^{m341} has a T→A substitution at residue Y_{761}, which results in early termination.
- The coatomer subunits β, β', ε, δ, γ2 and ζ2 are up-regulated within the developing notochord at early stages and shut down by 24 hpf.
- *COP*β, *COP*β', *COPε*, *COPδ*, *COPγ2* and *COPζ2* are all maintained within the undifferentiated notochord of COPI mutant embryos at 28 hpf.
- The COPI subunit *COPζ* is not up-regulated within the notochord at early stages and is not present within the notochord of COPI mutants at 28 hpf.

Chapter Six

Effect of COPI Loss on ER and Golgi Structure

6 Effect of COPI loss on ER and Golgi Structure

In this chapter the effects of loss of COPI function will be examined. Specifically, the effect of COPI loss on the structure of the ER and the Golgi. Using fluorescently tagged proteins, localised to either the Golgi or the ER to visualise these organelles, the effect of COPα loss has been examined through MO knock-down.

6.1 Introduction

Treatment with BFA and disruption of COPI function in the mutants *sly, hap* and *dop* causes a breakdown in the structure of the Golgi and the ER (Coutinho et al., 2004). The fusion proteins Cal-CFP and GalT-YFP localise to the ER and the Golgi respectively. Cal-CFP localises to the ER through the function of the Calreticulin KDEL ER retrieval sequence (Fliegel et al., 1989; Munro and Pelham, 1987). GalT-YFP localises to the Golgi through the function of the N-terminal 81 amino acids of 1, 4-galactosyltransferase (Llopis et al., 1998; Yamaguchi and Fukuda, 1995). By injecting linearised DNA vectors encoding these fusion proteins, the structure of both the Golgi and ER could be observed in individual living cells. Injection of the COPα MO or treatment with BFA in vector injected embryos enabled visualisation of ER and Golgi structure in COPI defective cells, including notochord cells.

6.2 Loss of Coatomer in Notochord and Muscle Cells

By 32 hpf, the phenotype of COPα MO injected embryos is apparent and it is therefore at this stage that the structure of the Golgi and the ER was compared to un-injected embryos. Visualisation of wild type notochord cells, in eight MO injected and ten wildtype embryos at this stage demonstrated distinct localisation of both Cal-CFP and GalT-YFP in regions resembling the ER and the Golgi respectively. This localisation is concentrated within a relatively small area of the cell, presumably due to the vacuolation of the notochord cell as it differentiates, around an area of low fluorescence that is most likely the nucleus. It is around this area that the majority of ER localised Cal-CFP is concentrated, with GalT-YFP concentrating in one large distinct body. Visualisation of ER and Golgi structure is more difficult on morphant cells, due to their small size as a result of lack of differentiation. However, close observation demonstrates that the Golgi localised GalT-YFP occupies a large body that occupies almost the entire cell, suggesting Golgi swelling, and the ER localised Cal-CFP is diffused throughout the cell, suggesting a breakdown of the ER (**Figure 6.1**).

The structure of the Golgi and ER in wild type muscle cells is comparable to the structure observed in notochord cells. Both the Golgi and the ER are localised around circular bodies resembling the multiple nuclei of the cell. Both the ER and the Golgi are smaller within muscle cells, which may be a reflection of the increased secretory demand of notochord cells. ER and Golgi are also less localised, occurring in discrete bodies spaced around the nuclei, possibly due to the lack of vacuolation. There is, as in notochord cells, no overlap of the Cal-CFP and the GalT-YFP, which befits localisation to the Golgi and ER respectively. In COPα MO injected embryos,

the affect on Golgi and ER structure is more evident in the larger muscle cells. Loss of COPI function results in a breakdown of Golgi structure and diffusion throughout the cell. Though large Golgi bodies remain, the entire cell demonstrates a much higher background level of GalT-YFP fluorescence, indicating loss of Golgi organisation. In morphant cells, Cal-CFP fluorescence is much lower and localised to several diffuse areas. Thus, there appears to be both an enlargement and a breakdown of the Golgi and a breakdown and loss of the ER (**Figure 6.1**).

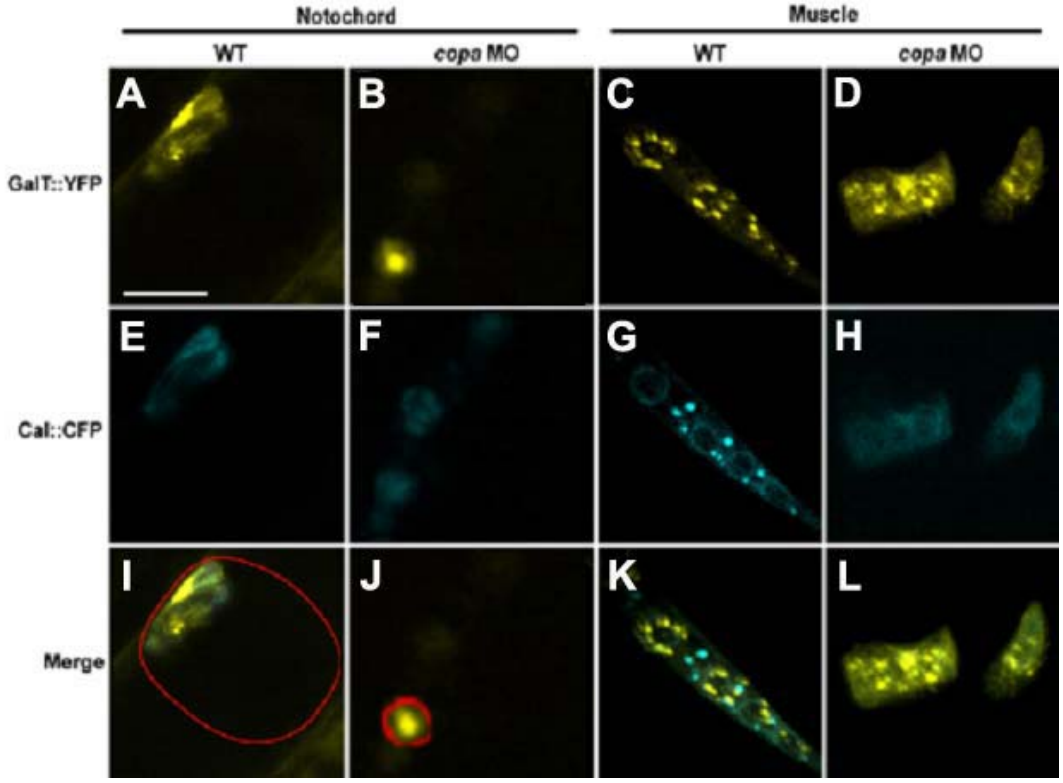


Figure 6.1 Structure of Golgi and ER in normal and COPI loss of function embryos.

Structure on the Golgi (A-C) and ER (E-H) in wild type notochord (A, E and I), COPI loss of function notochord (B, F and J), wild type muscle (C, G and K) and COPI loss of function muscle (D, H and L) cells. Red boundary in panels I and J marks the boundary of the notochord cells.
Bar (A) represents 20μM in (A, C, D, E, G, H, I, K and L) and 40μM in (B, F and J).

6.3 Effect of COPI Loss of Function Over Time

One of the benefits of using confocal techniques is that one can visualise fine structure in living cells through the use of specifically localised fluorescently tagged proteins. As such, the ER and Golgi localised fluorescent vectors were used to examine the effects of COPI loss of function over time. Embryos were injected with linearised vectors prior to 4 cell stage and raised to under normal conditions until 18 somite stage. At this point, embryos were changed to 1.8μM BFA in embryo water and were then raised under normal conditions until visualised. Thus, initial COPI activity was unaltered by COPI loss and GalT-YFP and Cal-CFP are able to localise properly in the early embryo. Addition of 1.8μM BFA at 18 somite stage has been shown to generate embryos with comparable phenotype to the COPI mutants (Coutinho et al., 2004). Addition of BFA was monitored in six embryos over a three hour period, beginning at 28 hpf, at which time the phenotype was not fully penetrant, so that Golgi and ER structure were not yet completely disrupted. A muscle cell from one embryo and three unspecified tail cells from a separate embryo are shown in **Figure 6.2**.

In both groups of cells, the Golgi and the ER structure initially resembled wild type embryos. GalT-YFP is localised in discrete bodies surrounding the nuclei. Cal-CFP is present throughout the cell, but concentrated in distinct, regular bodies around the nucleus. After one hour (29 hpf), there is an obvious breakdown and fragmentation of the ER and GalT-YFP localisation becomes more diffuse, with the Golgi bodies reducing in number. At two hours, Cal-CFP is more diffuse and remains localised in only a small region surrounding the nucleus. At this point the level of GalT-YFP fluorescence within the cell is higher and the Golgi bodies have

become fewer. By three hours, the ER is a thin body surrounding the nucleus, with Cal-CFP diffused throughout the cell. CFP fluorescence is much lower and the ER is reduced is size and highly fragmented and fractured. GalT-YFP has become diffused highly throughout the cell and there are few remaining Golgi bodies. Localised fluorescence of GalT-YFP within the Golgi bodies is less than at 28 hpf (**Figure 6.2**).

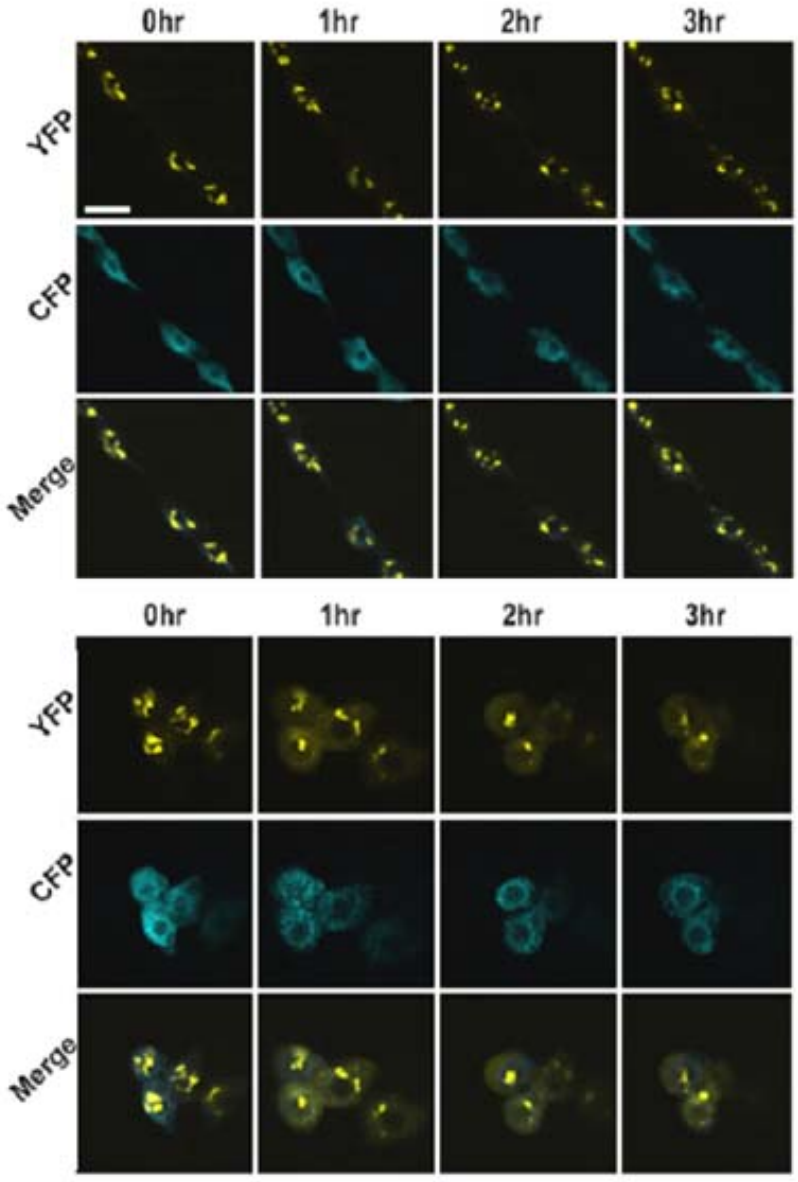


Figure 6.2 Effect of COPI loss of function on the localisation of ER-CFP and Golgi-YFP.

Top panel: Effect of loss of COPI function in muscle cells on Golgi structure (top row) and ER structure (middle row). Merged displayed on bottom row.
Bottom panel: Effect of loss of COPI function in undefined trunk cells on Golgi structure (top row) and ER structure (middle row). Merged displayed on bottom row.
Time 0 represents 28 hpf through to 3 hr, which represents 31 hpf.
Bar (top left micrograph) represents 20 μm.

6.4 Discussion

Examination of both Golgi and ER structure through the use of ER localised, CFP tagged, Calreticulin and Golgi localised, YFP tagged 1,4-galactosyltransferase has demonstrated that loss of COPI function, through MO knock down of COPα, results in disruptions to the Golgi and ER structure. Loss of COPI function, through either MO knock-down of COPα or treatment with BFA, results in a breakdown of the ER and a dispersal of Cal-CFP throughout the cell. GalT-YFP fluorescence becomes highly diffuse under conditions of COPI loss and Golgi bodies decrease in number and increase in size. These observations fit with a model of loss of transport between the Golgi and the ER, as fits with a loss of COPI function. The major role of COPI is in retrograde transport, in the Golgi and from the Golgi to the ER. Under normal conditions, ER resident proteins are captured in forming vesicles and transported to the Golgi, to then be returned to the ER through identification of target sequences, such as those within calreticulin, through the action of COPI vesicles. These vesicles also function in maintaining Golgi components in their proper localisation. Under conditions of COPI loss, retrograde transport is lost, ER resident proteins are transported to the Golgi and lost from the ER and Golgi components are lost from their proper Golgi location, again, due to a lack of retrograde transport functioning within the Golgi network.

Observations of Cal-CFP localisation support this, where initial fluorescence is localised to small bodies around the nucleus, only to fragment and diffuse throughout the cell as loss of COPI function takes effect. Such fragmentation and diffusion is likely to be due to the dispersal of Cal-CFP through the Golgi bodies and beyond, being lost to the cytosol of the cell as the ER breaks down. The localisation of GalT-

YFP also fits with the role of COPI and predictions on the effect of COPI loss on secretory network structure. A loss of retrograde transport within the Golgi results in a loss of Golgi components to the cytosol as well as the incorporation of ER resident proteins to the Golgi. Observations of GalT-YFP localisation demonstrate both a fusion of individual bodies of GalT-YFP fluorescence as well as a diffusion of fluorescence throughout the cell. Such observations can be explained through the fusion of individual Golgi bodies, due to an increase in the influx of material from the ER which is incapable of undergoing retrograde transport as well as the loss of retrograde transport within the Golgi, so that individual compartments are lost and incorporated into one large abnormal Golgi body. The diffusion of GalT-YFP throughout the cell is likely due to the loss of Golgi components to the cytosol due to both a loss of retrograde transport, so that Golgi components are secreted from the network, and the breakdown of Golgi bodies under the abnormal stress, releasing their contents into the cytosol. Hence, loss of COPI function results in a breakdown and loss of the ER, with dispersal of ER components throughout the Golgi and cytosol. Golgi bodies become fused and Golgi contents becomes dispersed throughout the cell under conditions of COPI loss.

6.5 Summary

- In wild type cells Cal-CFP and GalT-YFP localise to organelles that closely resemble the ER and the Golgi respectively.
- Notochord cells lacking COPI function are much smaller than wild type notochord cells due to a lack of vacuolation.

- In cells lacking COPI function, at 32 hpf, there is a dispersal of GalT-YFP throughout the cell and a reduction in the number of highly fluorescent bodies.
- At 32 hpf, COPI deficient cells show a loss of Cal-CFP fluorescent bodies and low levels of Cal-CFP throughout the cell.
- COPI loss of function causes a breakdown of the ER and dispersal of its contents throughout the cell combined with a reduction in the number of Golgi bodies and dispersal of Golgi contents into the cytosol.
- COPI deficient cells have ER and Golgi structure resembling wild type cells at 28 hpf, but by 31 hpf show breakdown of ER and Golgi structure.

Chapter Seven

Regulation of COPI Expression

7 Regulation of COPI Expression

In this chapter I will examine some of the questions raised by the observations of the previous section. To that end, I examined the conditions under which COPI subunit expression is maintained. Specifically, I examined the role of several components of the unfolded protein response (UPR) in development, in the normal expression of COPI subunits and in their maintained expression in the COPI mutants. A brief review of unfolded protein response and its role in both stress response and in normal secretory network maintenance is provided.

7.1 Introduction

Loss of COPI function in response to treatment with BFA results in a breakdown of secretory organelle structure, a loss of proper secretion and protein folding, which thus leads to an increase in the build up of unfolded protein. To ensure proper folding of intra-organelle, secretory and transmembrane proteins, the ER hosts a set of specialised protein folding components that promote and assist the proper folding and act to prevent the formation of protein aggregates. During normal secretion these components are lost from the ER through forward movement along the secretory pathway, though they are then transported back to their proper localisation though retrograde action by COPI (Letourneur et al., 1994; Sonnichsen et al., 1996). Effects that alter the delicate balance of ER components can lead to disruptions in protein folding and a build up of harmful protein aggregates. Loss of COPI, which leads to a loss of retrograde transport, causes a loss of ER components

and hence leads to a loss of protein folding and a build up of protein aggregates. Increases in the protein folding load, due to elevations in the synthesis of secretory proteins, can lead to the increase in the requirement for protein folding and hence an increase in unfolded proteins and protein aggregates, as can treatment with small molecules, such as tunicamycin, which blocks *N*-glycosylation of newly synthesised protein (Duksin and Bornstein, 1977; Kawahara et al., 1997). The requirement of eukaryotic cells to adapt to such demands and to avoid the detrimental affects of unfolded protein build up and the accumulation of protein aggregates has led to the evolution of an adaptive response to limit the accumulation of unfolded proteins within the ER. Originally described through the study of the SV40T antigen that was targeted to the ER (Kozutsumi et al., 1988), this signalling response has become known as the Unfolded Protein Response (UPR) (for reviews see (Kaufman et al., 2002; Patil and Walter, 2001)).

The initial characterisation of the components of the UPR was performed in the budding yeast *Saccharomyces cerevisiae*, where genetic screens identified three proteins involved in the transduction of signals from the ER to the nucleus. Three proteins, Ire1p, Hac1p and Rlg1p were identified as playing a vital role in the process of UPR signalling. Ire1p is a transmembrane serine/threonine kinase with three functional domains. The amino-terminal domain resides within the ER lumen, where it acts to sense the level of unfolded protein with the ER (Cox et al., 1993). The accumulation of unfolded protein within the ER causes dimerisation of Ire1p, which results in trans-autophosphorylisation by its cytosolic kinase domain (Shamu and Walter, 1996; Welihinda and Kaufman, 1996). This then causes the activation of Ire1p's, carboxy-terminally located, site specific endoribonuclease, the substrate for which is *Hac1* mRNA. *Hac1* encodes a basic leucine zipper (bZIP) transcription

factor that acts to induce the expression of UPR target genes (Kawahara et al., 1997). *Hac1* is constitutively expressed in an un-translated from, such that, although *Hac1* mRNA is continually present, the encoded Hac1p protein is not detected under normal conditions (Cox and Walter, 1996). The *Hac1* un-translated mRNA contains a 252 nucleotide intron located near the 3' end that, when present, acts to block the translation of the mRNA (Chapman and Walter, 1997; Cox and Walter, 1996; Kawahara et al., 1997). Removal of this intron results in an mRNA encoding a 10-fold more effective transcription factor and an mRNA that is more efficiently translated (Mori et al., 2000; Ruegsegger et al., 2001). In response to activation of the UPR, Ire1p acts directly upon *Hac1* mRNA to cleave at two specific sites (Sidrauski and Walter, 1997). The 5' and 3' *Hac1* mRNA fragments are then joined through the action of the tRNA ligase Rlg1p (Sidrauski et al., 1996). The splice-activated form of *Hac1* mRNA is then translated to produce the Hac1p protein, which is translocated to the nucleus, where it activates the expression of UPR genes through direct binding to the upstream Unfolded Protein Response Element (UPRE) (Cox and Walter, 1996; Kawahara et al., 1997; Mori et al., 1996). The UPRE is both necessary and sufficient for up-regulation in response to the UPR (Mori et al., 1998; Mori et al., 1992). Thus, in yeast, the action of Ire1p in splicing the *Hac1* mRNA regulates activation of the UPR (**Figure 7.1**).

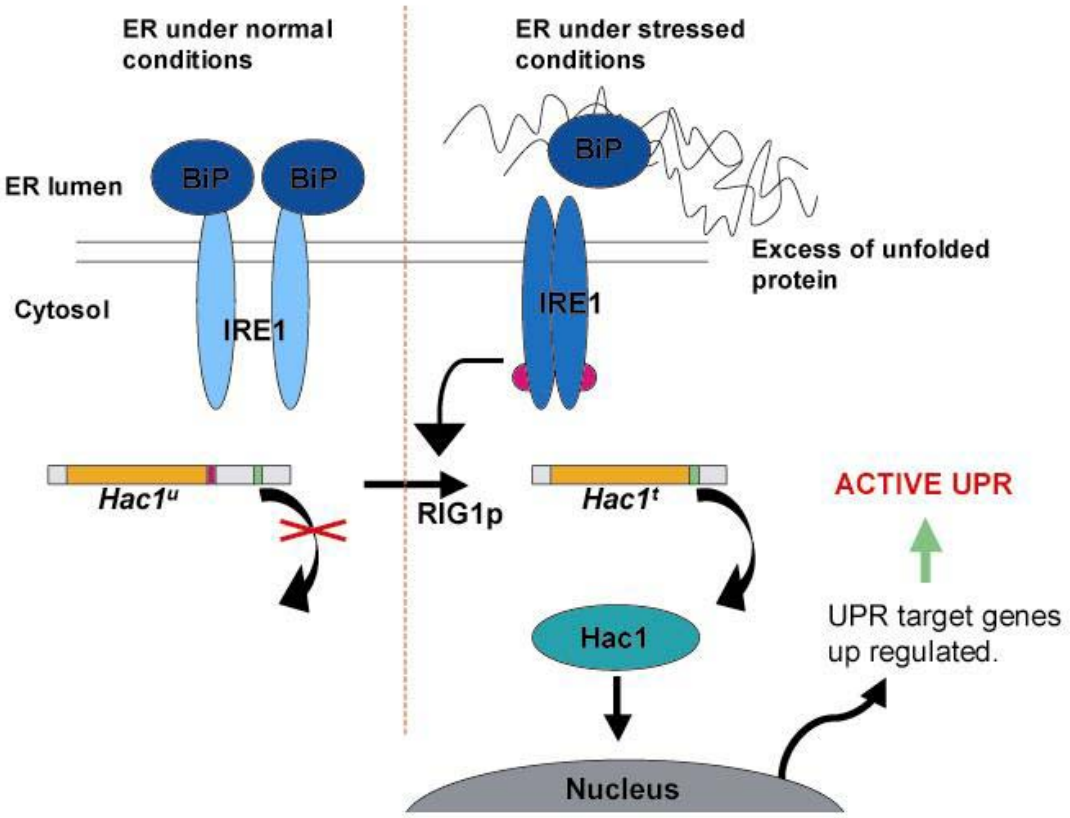


Figure 7.1 UPR activation in yeast.

In yeast, a build up of unfolded protein within the ER prevents inhibitory binding of BiP to IRE1. Under these conditions, IRE1 dimerises and self-phosphorylates, leading to is activation and the removal of the inhibitory intron from *Hac1*. *Hac1* is then spliced, where RIG1p acts to ligate the exons of *Hac1*. This more efficiently translated mRNA is then translated to form the Hac1 protein. This protein then acts to directly up-regulate UPR target genes.

Ire1p and Hac1p act in a linear pathway to active UPR response genes under conditions of ER stress. Ire1p acts to detect the level of unfolded protein within the ER and under conditions of high unfolded protein load, splices *Hac1p* which then up-regulates the UPR response genes. The key factor in Ire1p's ability to sense the level of ER stress is the ER chaperone protein BiP. Bip/GRP78 has long been a marker of UPR activation, since it is rapidly upregulated under conditions of UPR activation (Kaufman, 1999) and acts in the ER to chaperone proteins during proper protein folding (for review see (Ma and Hendershot, 2004)). Under normal conditions, Ire1p exists in a monomeric form through the binding of BiP to its ER lumen exposed surface, but under conditions of ER stress, where there is a build up of unfolded protein, more of the available BiP becomes associated with chaperoning unfolded proteins through binding to the exposed hydrophobic regions (Bertolotti et al., 2000). This reduces the level of BiP available to bind to Ire1p, thus resulting in the oligomerisation of Ire1p. So, the activation of the UPR in yeast is regulated by the ER protein chaperone BiP, which, under normal conditions, acts to prevent oligomerisation and hence activation of Ire1p through direct interaction. However, under conditions of ER stress, where there is a build up of unfolded protein, BiP is engaged in chaperoning the increased unfolded proteins, leaving Ire1p unbound and allowing oligomerisation, trans-autophosphorylation and activation.

The UPR in yeast is a simple linear pathway, signalling increases in unfolded protein load through BiP, Ire1p and Hac1p. However, UPR is more complicated in higher eukaryotic organisms. Though much of the yeast UPR system has been conserved during evolution, there are significant differences in even the conserved components. There are two homologues of yeast IRE1 in mammalian genomes, termed IRE1α and IRE1β (Niwa et al., 1999; Tirasophon et al., 1998; Wang et al.,

1998). Interestingly, Ire1α is expressed in many cells and organs where Ire1β is limited to expression within the epithelial cells of the gut (Urano et al., 2000b). The mechanism of IRE1 action in higher eukaryotes is similar to its action in yeast, oligomerising in conditions of high unfolded protein load due to a reduction in the level of available BiP, and self activating through trans-autophosphorylation. In mammals, the mRNA substrate for Ire1 has been identified as the bZIP transcription factor X-box binding protein 1 (Xbp1) (Calfon et al., 2002; Shen et al., 2001; Yoshida et al., 2001). Activation of Ire1 results in the removal of a 26 nucleotide intron from the *Xbp1* mRNA to generate a potent transcriptional activator similar to *Hac1p* in yeast. However, the loss of both Ire1α and Ire1β does not reduce the transcription activation in response to UPR of several UPR responsive genes (Lee et al., 2002; Urano et al., 2000a; Urano et al., 2000b). However, loss of Ire1α has been reported to result in a transcription defect in mouse fibroblasts, which can be complemented through expression of the spliced *Xbp1* (Lee et al., 2002). Hence, the Ire1 response controls only a subset of UPR genes. In support of this, two other UPR signalling pathways have been characterised, involving the protein ATF-6 (Yoshida et al., 1998) and the protein PERK (Harding et al., 1999; Shi et al., 1998).

ATF-6 is a constitutively translated ER membrane protein that remains inactive during normal conditions. However, under conditions of increased unfolded protein load the cytosolic domain of ATF-6 is cleaved in a step requiring the site 2 protease (S2P) (Ye et al., 2000), from the ER and translocated to the nucleus where it activates the expression of genes including *BiP* and *Xbp1* (Haze et al., 1999; Lee et al., 2002; Yoshida et al., 2000). Increased transcription of *Xbp1* by ATF-6 provides more substrate for activated Ire1, which then results in a positive feedback loop for the UPR. Further to this, ATF-6 offers a more rapid response to increases in unfolded

protein load, since it requires only cleavage and translocation to function in up-regulating UPR genes, where Ire1 action requires not only splicing of *Xbp1*, but also translation. Interestingly, loss of either Ire1α and Ire1β or ATF-6 alone does not result in a loss of *Xbp1* expression in response to ER stress, suggesting that there is some overlap in the role of these two pathways in the UPR (Lee et al., 2002). Activation of ATF-6 cleavage in response to ER stress is also controlled through the action of BiP (Shen et al., 2002). In normal conditions, BiP binds to the ER lumen domain of ATF-6, preventing its transport to the Golgi complex. However, during periods of increased ER stress, when there is an increase in the presence of unfolded protein, available BiP is concerned with chaperoning the unfolding proteins leaving ATF-6 free to pass into the Golgi complex for proteolytic cleavage.

Activation of the UPR results in a notable reduction in the rate of translation. This slow down helps to protect cells from the fatal effects of unfolded protein build up by preventing the continued production of proteins under conditions that are non-conducive to proper protein folding. The identification of PERK as a transmembrane ER localised eIF2α kinase strongly implicated it as a key signalling element in the attenuation of translation in response to unfolded protein stress (Harding et al., 1999; Shi et al., 1998). Characterisation of PERK demonstrated that the ER luminal domain is considerably similar to the luminal domain of Ire1 and that the cystosolic domain shows the features of a Gcn2 kinase (Harding et al., 1999; Shi et al., 1998). Interestingly, the ER luminal domains of both Ire1 and PERK from both humans and *Caenorhabditis elegans* can be substituted for the luminal domain of yeast Ire1p and still illicit a UPR response (Liu et al., 2000), thus indicating that both PERK and Ire1 act through a common mechanism. So, it is likely that activation of the UPR results in oligomerisation and trans-autophosphorylation, which results in activation of the

Gcn2 kinase domain that acts to phosphorylate the translation initiation factor eIF2α. Cells lacking PERK demonstrate greater levels of Ire1 phosphorylation upon activation of the UPR, indicating that PERK acts to prevent the increase in unfolded protein load. The ability of cycloheximide to partially inhibit this increase in UPR activation supports the role of PERK in blocking translation (Harding et al., 2000b). The activation of PERK in response to accumulation of unfolded protein in the ER is, like Ire1 and ATF-6 controlled by BiP (Bertolotti et al., 2000; Liu et al., 2000). Under normal conditions BiP binds to the ER luminal domain of PERK and prevents oligomerisation, with BiP becoming involved in chaperoning unfolded protein in times of ER stress, leaving PERK free to dimerise and trans-autophosphorylate, activating its cytosolic Gcn2 domain in a mechanism much like that or Ire1. However, approximately one third of UPR induced genes require the phosphorylation of eIF2α by PERK (Scheuner et al., 2001), indicating that PERK does not just function in the repression of translation during UPR. The phosphorylation of eIF2α by PERK results in the preferential translation of ATF4, which in turn results in the up-regulation of UPR genes, including *CHOP* (Harding et al., 2000a; Scheuner et al., 2001). Indeed, cells lacking PERK or eIF2α lack almost all *CHOP* expression, though this is not the case in cells lacking both *Ire1* isoforms (for an overview of UPR in vertebrates see **Figure 7.2**).

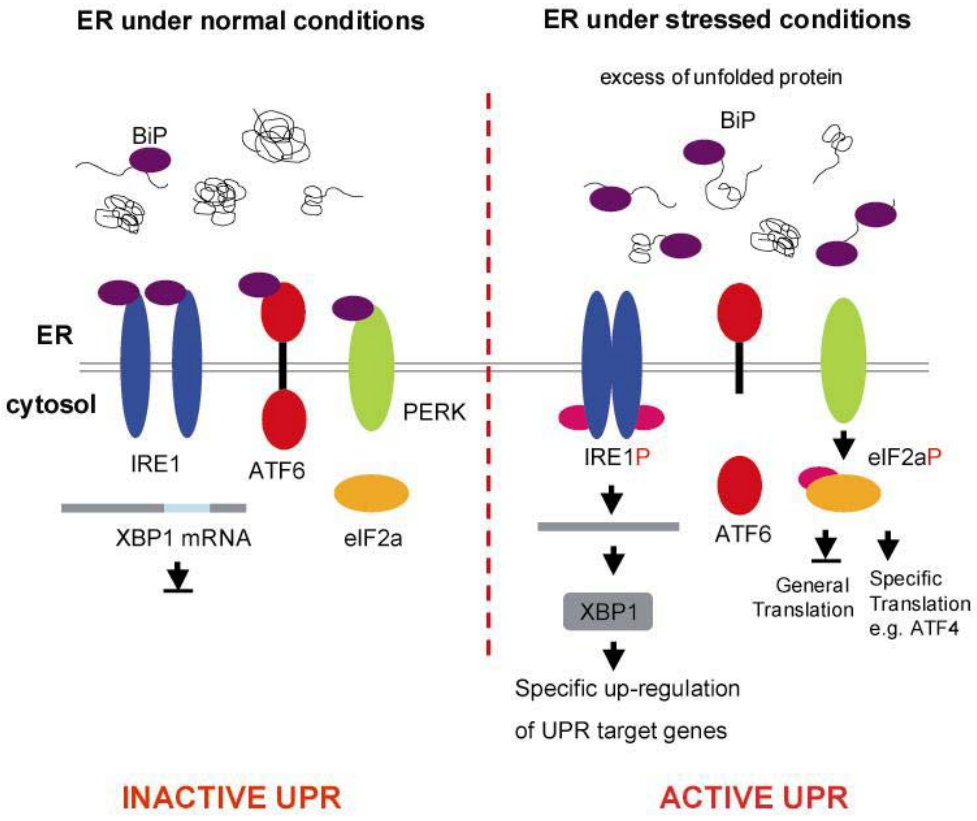


Figure 7.2 Activation of the UPR in higher organisms.

Build up of unfolded protein in higher eukaryotes results in a loss of BiP mediated inhibition of IRE1, ATF-6 and PERK. Under these conditions, IRE1 dimerises, causing self-phosphorylation, which in turn leads to the activatory slicing of *Xbp1* and the efficient translation of functional XBP1 that directly acts in up-regulating UPR target genes. ATF-6 cleaves, releasing the cytosolic tail that then acts to up-regulate UPR target genes, including *Xbp1*. Activation of PERK results in the phosphorylation of eIF2α, which in turn acts to down-regulate translation, relieving the protein load, but also results in the specific translation of certain genes e.g. *ATF4*.

The ultimate fate of cells that are unable to resolve the unfolded protein load after entering the UPR is death through apoptosis. Extended treatment of cells with tunicamycin results in the activation of the ER membrane caspase-12, causing apoptosis (Harding et al., 2000b). This decision is most likely made after the cell has entered cycle arrest, when the UPR has resulted in a sufficient decrease in translation of Cyclin D1 to result in arrest at G1 phase (Brewer and Diehl, 2000). The first cell death promoting factor to be identified was CHOP, a pro-apoptotic transcription factor (Friedman, 1996; Zinszner et al., 1998). The induction of *CHOP* is complicated; over-expression of Ire1 is able to induce its expression, with expression of a dominant negative Ire1 resulting in some loss of expression (Wang et al., 1998). However, a lack of Ire1 does not result in complete loss of *CHOP* expression upon activation of the UPR where loss of PERK results in a much more dramatic loss of *CHOP* (Scheuner et al., 2001). Such evidence suggests that *CHOP* expression may be controlled by PERK activation with Ire1 able to induce expression, possibly though interactions that result in PERK activation. Though CHOP has a key role in inducing the expression of apoptosis genes, it is not required for apoptosis. Cells lacking PERK lack almost all CHOP, but are hypersensitive to ER stress and enter apoptosis more readily than normal cells, thus indicating that other factors are involved in UPR induced apoptosis.

Much information concerning the role of the UPR under normal physiological conditions has been defined through the use of *C. elegans*. The UPR in *C. elegans* involves the same systems as described above, utilising the signalling molecules Ire1, ATF-6 and PERK (Calfon et al., 2002; Shen et al., 2001). Loss of either *ire-1* and *pek-1*, the *C. elegans* homologues of *Ire1* and *PERK* respectively, causes no discernable difference when compared to wild type animals. However,

loss of both *ire-1* and *pek-1* together resulted in developmental arrest and intestinal necrosis at the L2 stage in animals raised under normal type conditions (Shen et al., 2001). This phenotype in the intestinal cells, which demonstrate an increase in the synthesis of secreted protein, suggests that the UPR may function during development to meet the increased stresses placed on cells that require an increase in protein folding and secretion as part of their development. Recent work has demonstrated that the UPR, specifically *ire-1* but not *pek-1* and *atf-6*, are necessary for the proper movement of AMPA-type glutamate receptor subunits through the secretory pathway in neurons (Shim et al., 2004). In animals lacking *ire-1* and raised under normal conditions, the GLR-1 subunit is retained within the ER. In support of this, the same study demonstrated that *Xbp1* is up-regulated in neurons during normal development. Thus suggesting that the UPR plays an essential role during normal development in *C. elegans*.

In this section, the role of the UPR during development of the zebrafish will be examined, specifically, the role of the UPR in relation to coatomer expression and function during development. In this section I will put forward evidence to suggest that the UPR is not simply a stress response, but a general mechanism functioning during normal development to meet the increased rate of secretion and translation in specific tissues as they develop and differentiate. I will also attempt to link the UPR to expression of coatomer, proposing the UPR as a possible mechanism for both the normal expression of coatomer during development as well as the maintained expression of coatomer under conditions of COPI loss of function.

7.2 Expression of COPI in Undifferentiated Notochords

COPα mRNA is maintained in the undifferentiated notochord of *sny* embryos at 28 hpf, as is *ehh*. The early notochord marker *ehh* is also maintained within the undifferentiated notochord of *sly* embryos, which lack the laminin γ1 chain, thus *ehh* is maintained simply due to a lack of proper notochord development rather than the lack of any specific gene function. To examine the possibility that *COPα*, and other COPI subunits, may be maintained due to the specific loss of COPI function in the COPI mutants or due to the failure to fully differentiate a proper notochord, the expression of *COPα* and *ehh* was examined in *sly* embryos, which fail to differentiate notochord but still have a functional COPI complex.

Expression of *ehh* is maintained specifically within the notochord of both *sny* and *sly* embryos, demonstrating that the notochord fails to differentiate properly in both mutants. However, *COPα* expression is maintained only in the notochord of *sny* mutants, indicating that it is the specific loss of COPI function that causes the maintenance of *COPα* expression in COPI mutants beyond the normal temporal limitations (**Figure 7.3**).

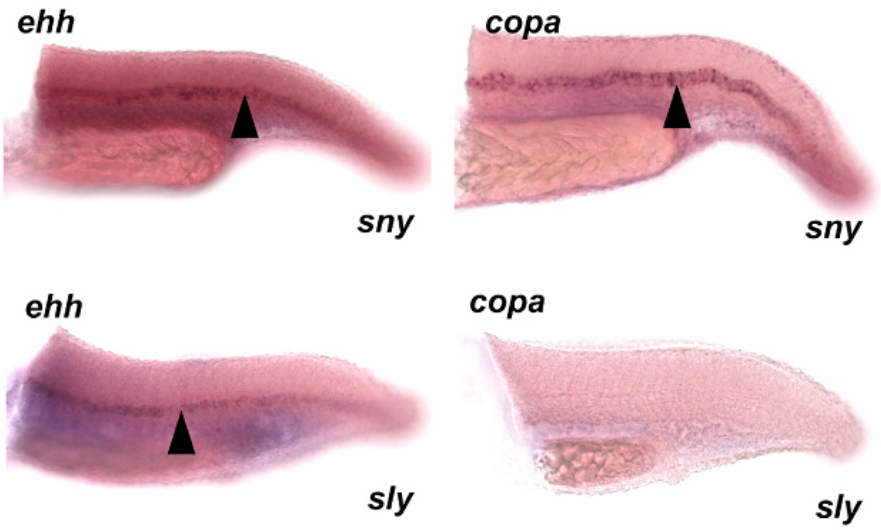


Figure 7.3 Expression of *COPα* and *ehh* in *sny* and *sly* embryos.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top.
ehh is maintained in the undifferentiated notochords of both *sny* and *sly* mutants at 28 hpf. *COP*α is maintained only in the undifferentiated notochords of *sny* embryos. Thus, *COP*α is maintained only in notochords lacking COPI function, and is not maintained just as a results of lack of notochord differentiation.

7.3 Identification of UPR Components in Zebrafish

Using publicly available EST and genomic information, combined with cDNA sequence for UPR components from mouse, full length cDNA sequence was generated for the zebrafish components of the UPR. In this way, a single IRE1 homologue was identified as being 2813 bases long, predicting a protein of 931 aa that shows 58% identity to mouse IRE1α and 52% identity to mouse IRE1β. Full length cDNA sequence was generated for zebrafish ATF-6, PERK and BiP of 1797, 3006 and 2347 bases respectively. These encoded proteins of 560, 952 and 650 aa that show 41%, 58% and 89% identity to the mouse AFT-6, PERK and BiP respectively. The identified zebrafish *Xbp1* sequence was 1696 and 1675 in its inactive and active form respectively. These encoded proteins of 263 and 383 aa demonstrating identities of 56% and 47%, relating to the inactive and active forms respectively.

7.4 Activation of the UPR During Development and in COPI Mutants

Using the generated full length cDNA sequence for *BiP*, primers were designed to amplify a 1357bp fragment from wild type cDNA. From this, an *insitu* riboprobe for *BiP* was synthesised. This probe was then used to examine the expression of *BiP* in staged wild type embryos; at approximately 4 cell stage, shield stage, tail-bud stage, 5 somite stage, 12 somite stage, 24 hpf and in the three coatomer mutants; *sny*, *hap* and *dop* at 28hpf. *BiP* is considered to be one of the earliest induced components of the UPR and the encoded protein is a major

controller of activation of the UPR. As such, *BiP* is one of the most commonly used makers of UPR activation and in this instance, *BiP* expression is used to examine where and when the UPR is active.

7.4.1 Expression of BiP mRNA During Development

High levels of *BiP* at the 4 cell stage indicates that *BiP* is maternally expressed. This expression continues through to shield stage at which point *BiP* is expressed ubiquitously. By tail-bud, *BiP* is still ubiquitous, though at very low levels. However, by this stage there is a specific up-regulation of expression within the chordamesoderm. This chordamesoderm/notochord localised up-regulation continues through to 12 somites. Elevated levels of expression are also detected in the developing brain and hatching gland during somitogenesis. By 24 hpf expression is returned to low levels of ubiquitous expression throughout the embryo with the exception of the most posterior tip of the developing notochord. Thus, the expression of *BiP*, a widely used marker of UPR activation, closely resembles the expression of the coatomer subunits α, β, β', ε, δ, γ2 and ζ2 (**Figure 7.4**).

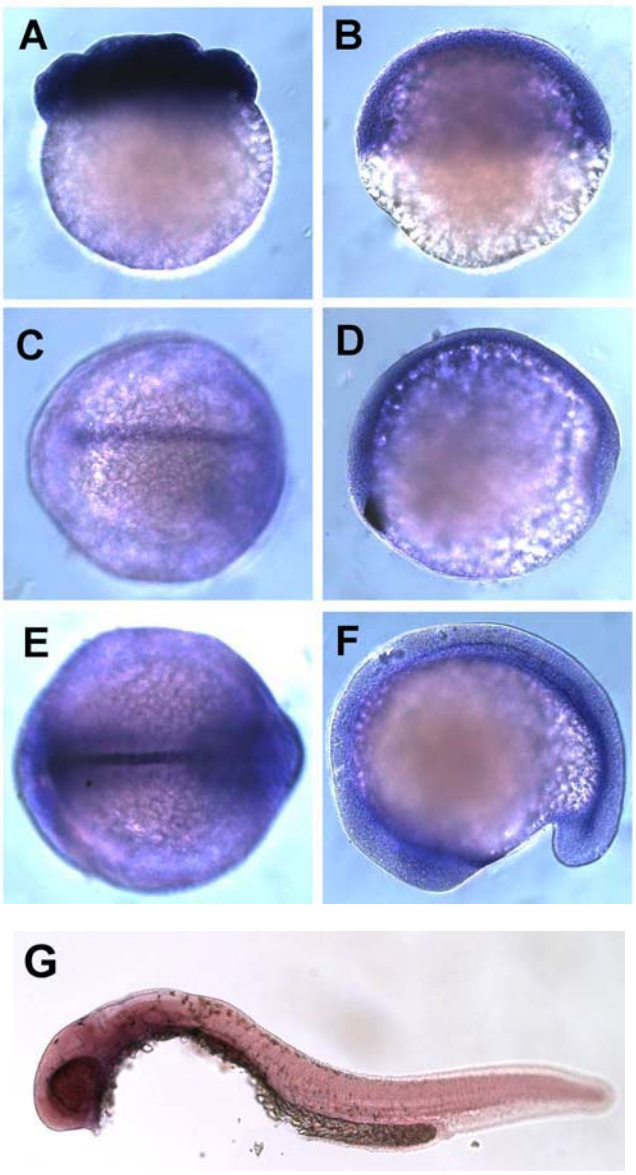


Figure 7.4 Staged expression profile of the UPR marker *BiP*.

At (**A**) 32-cell, (**B**) shield, (**C**) tailbud dorsal view, (**D**) tailbud lateral view, (**E**) 5-somite dorsal view, (**F**); and lateral view, anterior to the left, of 14 somite and (**G**) 24 hpf. (**A**) High level of expression at 32 cell stage demonstrates that *BiP* is provided maternally. At shield stage (**B**) expression is ubiquitous and at relatively high levels. By tailbud (**C** and **D**) stage, the ubiquitous expression is lower with the exception of specific up-regulation within the chordamesoderm. This is maintained through 5 somite (**E**) and 12 somite stages (**F**), with noticeably high expression in the developing brain at these stages. By 24 hpf (**G**) notochord specific expression has been extinguished and up-regulation is confined to the developing brain.

7.4.2 Expression of BiP mRNA in Coatomer Mutants

Comparison of *BiP* expression in both mutant and wild type sibling *sny*, *hap* and *dop* mutants demonstrated that, as observed with the majority of the COPI subunits, the chordamesoderm/notochord expression of *BiP* is maintained in 28 hpf *sny*, *hap* and *dop* embryos. In 28 hpf sibling wild type embryos, the chordamesoderm specific up-regulation is extinguished (**Figure 7.5**).

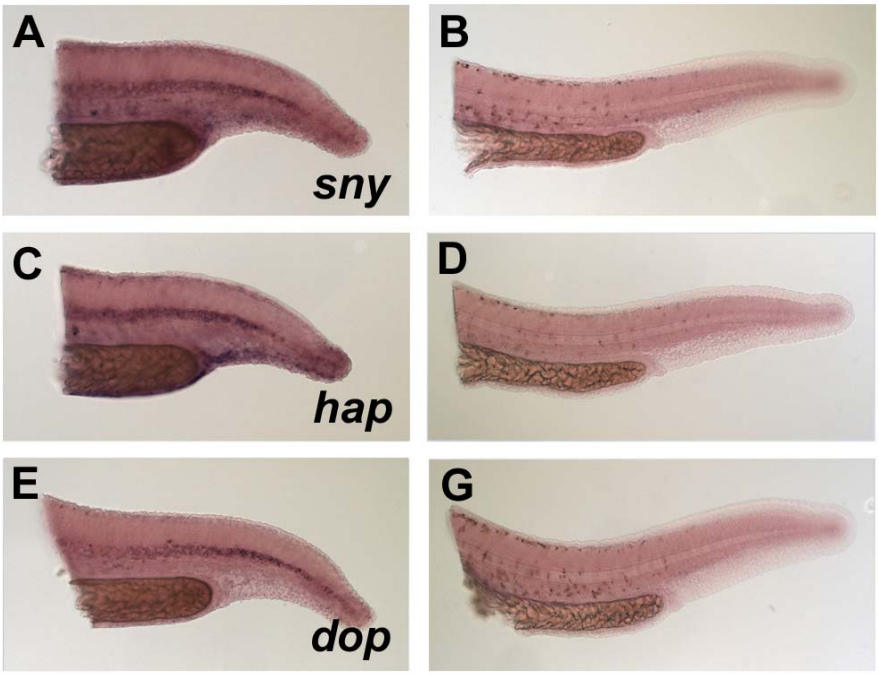


Figure 7.5 Expression profile of *BiP* in mutant and wild type embryos at 28 hpf.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top. Tails taken from mutant embryos (**A**, **C**, **E**) and wildtype siblings (**B**, **D**, **F**).
At 28 hpf, the Expression of *BiP* is maintained within the undifferentiated notochord of the COPI mutants *sny* (**A**), *hap* (**C**) and *dop* (**E**). The chordamesoderm/notochord specific expression of *BiP* is shut down in the properly differentiated notochord wild type siblings of *sny* (**B**), *hap* (**D**) and *dop* (**F**).

7.5 Morpholino Knock-Down of UPR Components

To examine the role to the UPR in development and in both the normal developmental expression of COPI and the abnormally maintained expression within COPI mutant embryos, MO's were designed against IRE1, ATF-6, PERK, BiP and XBP1. Using the generated cDNA sequences, MO's were directed against the ATG start of translation. Comparable doses of standard control MO (not shown) demonstrated no phenotype and resembled wild type uninjected embryos of equal stage.

7.5.1 Signalling Components of the UPR

Injection of 8ng of either an ATG targeted IRE1 or an ATG targeted ATF-6 resulted in comparable phenotypes. Both resulted in minor shortening along the A-P axis and a slight curling of the most posterior tail at 24 hpf. Neither IRE1 nor ATF-6 results in any observable defect in any other developmental process at this stage. In stark contrast to this, injection of 8ng of both ATF-6 and IRE-1 results in an obvious shortening of the embryo along the A-P axis at 24 hpf. At 24 hpf there are minor observable defects in notochord differentiation though there is not a complete failure to differentiate. By 48 hpf, embryos injected with both IRE1 and ATF-6 MO show a dramatic loss of notochord differentiation. These double injected embryos bear slight resemblance to the mutants *sly, hap* and *dop* at this stage. By 48 hpf, the double injection embryos show an even more dramatic shortening of the A-P axis, failure in notochord differentiation, with associated 'U' shape defects in somite

formation, major loss of melanophores, observable defects in the developing brain and the beginnings of wide spread necrosis in the majority of injected embryos. Despite the similarity of this phenotype to the COPI mutants, it was noticeably less severe, especially given that ATG targeted MOs should remove both zygotic and maternal transcripts (

Figure 7.6).

Since only one IRE1 isoform was isolated in zebrafish, a MO designed against the ATG of XBP1 was designed and used, since XBP1 is vital for IRE1 signalling and loss of XBP1 translation would block signalling from any uncharacterised IRE1 isoforms. Injection of 8ng of XBP1 resulted in a much more dramatic phenotype than either IRE1 or ATF-6 alone or IRE1 and ATF-6 combined. At 24 hpf, XBP1 injected embryos have a stark shortening along the A-P axis, loss of notochord differentiation combined with the associated 'U' shaped somite defect and major defects within the developing brain and head. In this way, XBP1 injected embryos also show similarity with COPI mutants, since the observed notochord defect resembles that observed in *sly, hap* and *dop*. However, the observed phenotype is more severe than that observed in the COPI mutants, since the COPI mutants show no obvious neural defects (**Figure 6.7**). Co-injection of 8ng of ATF-6 along with 8ng of XBP1 results in embryos demonstrating major defects in head structures, with considerable defects in somite structure, lacking any form of differentiated notochord and showing no discernable extension along the A-P axis. These defects result in a grossly amorphous embryo, which is further compounded by the beginnings of cell death throughout the embryo.

Injection of an ATG targeted MO against PERK produced a phenotype comparable to that observed in XBP1 injected embryos. Though in the case of

PERK, defects are obvious at a 4ng dose. In both cases there is a loss of proper notochord differentiation, the associated 'U' shape defect in somite structure and a loss of extension along the A-P axis. However, in the case of PERK, the observed phenotype in head and neural structures is less pronounced. Co-injection 8ng of ATF-6 with 4ng of PERK resulted in a more dramatic phenotype, generating embryos lacking almost all A-P extension and with severe malformations of the head, as well as lacking any notochord differentiation. Injection of 8ng of XBP1 with 4ng of PERK was more severe than co-injection of ATF-6, though the phenotypes were comparable. Co-injection of XBP1, ATF-6 at 8ng and PERK at 4ng resulted in mass necrosis and embryo death by 24 hpf. In the small percentage of embryos that do survive, there is a complete loss of proper morphology and structure. Injection of 4ng of IRE1 and ATF-6 and 2ng of PERK results in noticeable necrosis and a loss of both proper neural and notochord development. At this level of knockdown, the majority of embryos survive to 24 hpf but die by 36 hpf (**Figure 7.7**).

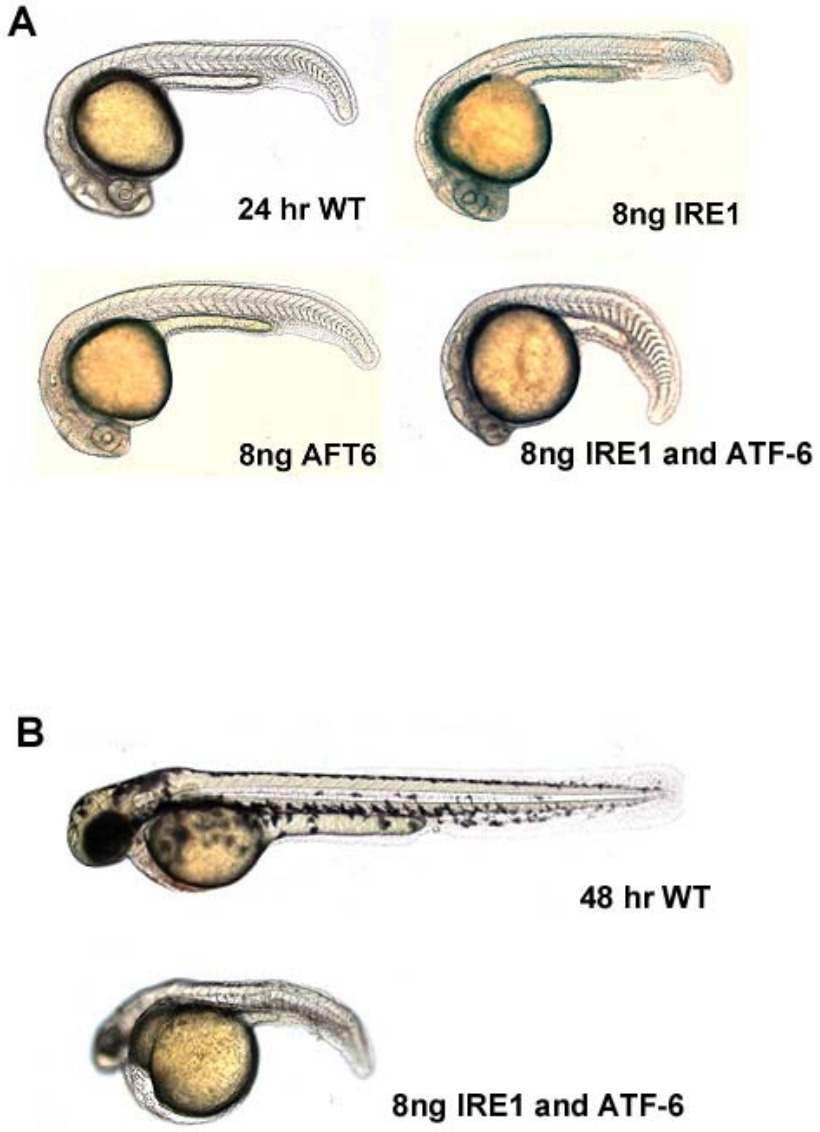


Figure 7.6 Embryos injected with ATG targeted MO's for IRE1 and ATF-6.

Lateral view, anterior to the left, dorsal to the top, of live 24 hpf embryos.
(**A**) 24 hpf WT embryos (top left) compared to embryos injected with 8ng IRE1 (top right), 8ng ATF-6 (bottom left) and IRE1 and ATF-6 combined (bottom right). Both IRE1 and ATF-6 alone result in no obvious defects, where combined injection results in an obvious A-P axis shortening, marked lack of notochord differentiation and minor neural defects.
(**B**) 48 hpf WT embryo (top) compared to 48 hpf embryos injected with 8ng of both IRE1 and ATF-6 (bottom). By 48 hpf, A-P axis reduction is more dramatic, somites have developed in a 'U' shape and there is no discernable notochord differentiation. In addition, neural defects are more obvious, with embryos displaying smaller heads and melanophores have failed to develop normally.

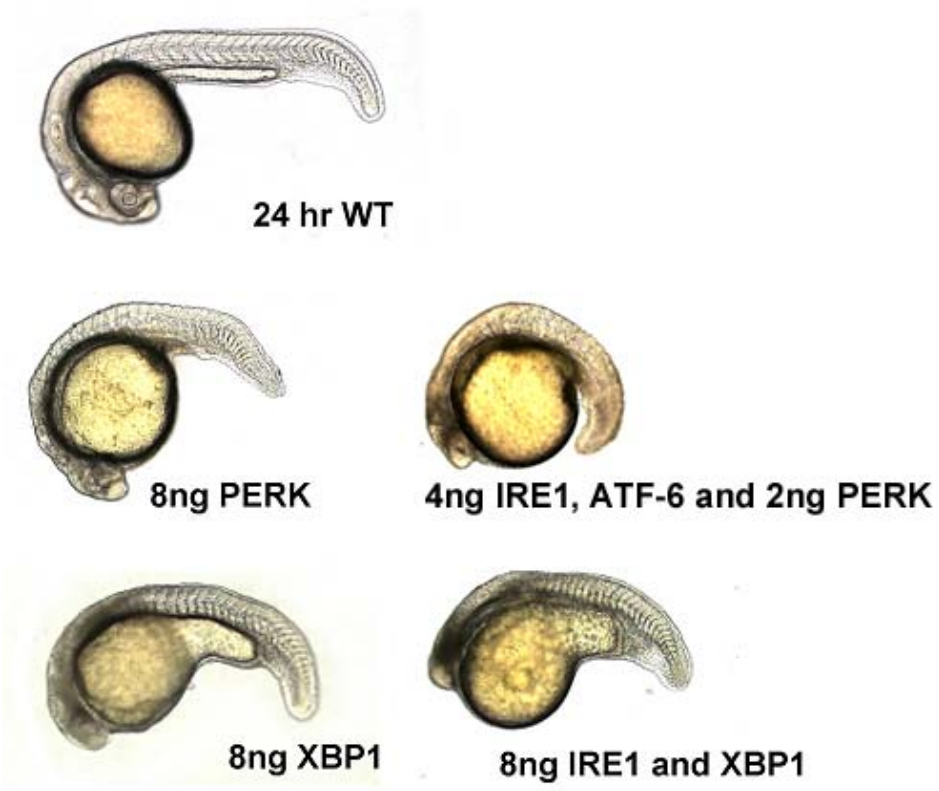


Figure 7.7 MO knockdown of PERK and XBP-1

Lateral view, anterior to the left, dorsal to the top, of live 24 hpf embryos.
Comparison of 24 hpf WT embryos (top) to PERK (middle left), PERK, IRE1 and ATF-6 (middle right), XBP-1 (bottom left) and IRE1 and XBP-1 (bottom right) MO injected embryos.
Loss of PERK alone or loss of PERK, IRE1 and ATF-6 combined, resulted in mild necrosis throughout the embryo at 24 hpf and a loss of proper neural and notochord development.
Loss of XBP-1 resulted in dramatic neural defects, obvious reductions in A-P axis extension and a complete lack of notochord development. Combined loss of IRE1 and XBP-1 is comparable to loss of XBP-1 alone, though moderately more severe.

7.5.2 the UPR 'Master Regulator' BiP

Injection of a MO targeted against the ATG start of translation of the zebrafish BiP resulted in both notochord and neural defects at 24 hpf. Injection of 2ng of MO against BiP resulted in a partially penetrant MO phenotype. A small percentage (~20%) of embryos demonstrated dramatic head defects, including defects in the developing eyes and stark neural abnormalities alongside a failure to differentiate notochord. In these embryos the posterior section of the tail loses any sense of structure and morphology and develops into a large growth of dying cells, though even in this, there can be observed some undifferentiated notochord cells. The less penetrant embryos show no obvious head abnormalities, developing eyes and showing no brain necrosis at 24 hpf. These embryos also demonstrate relatively normal extension along the A-P axis and a fairly well differentiated notochord, though there are minor defects in the posterior most limit of the developing tail where the tail curls and somites appear slightly compressed, suggesting a slight loss of A-P extension.

At higher doses of 8ng, knock down of BiP results in a much more obvious phenotype. At 24 hpf a small, but substantial, number of the injected embryos have died (~10%). The remainder all share a similarly expressive phenotype. These embryos are comparable to the affected embryos at 2ng, although they appear more severe. These embryos have drastic head defects, showing abnormalities in eye development, neural development and have abnormally small heads. These embryos also show a obvious lack of A-P extension, with the most penetrant embryos showing no tail beyond the yolk extension. Embryos also show the amorphous cell growth at the posterior end of the tail in many, but not all, cases. These embryos

demonstrate observable chordamesoderm and there appears to be differentiation to notochord. Further, the majority of 8ng BiP MO injected embryos show early signs of cell death and by 30 hpf all injected embryos have died through systemic necrosis (**Figure 7.8**).

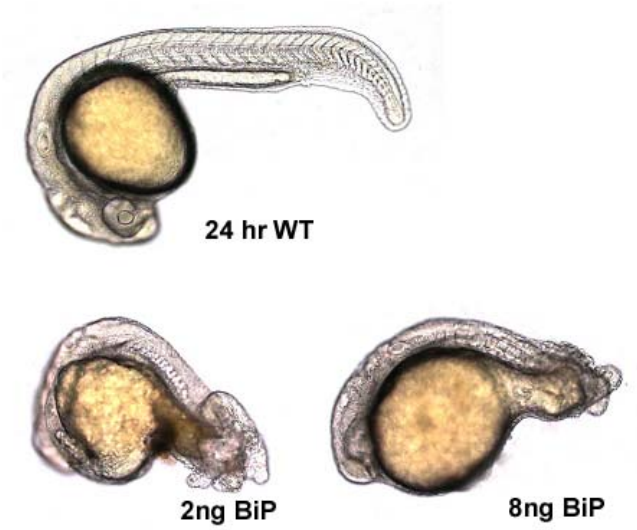


Figure 7.8 MO knock-down of BiP

Lateral view, anterior to the left, dorsal to the top, of live 24 hpf embryos.
Loss of BiP function results in deformations of the posterior tail tip, a lack of somite patterning and a loss of proper head development. Noticeably, both 8ng and 2ng injected embryos develop notochord and neural plate.

7.6 Role of the UPR in COPI expression

Using 8ng of XBP1 MO alone or 8ng of both XBP1 MO and ATF-6 MO in co-injected embryos, the expression of *BiP* and *COP*β' mRNA was examined under conditions of loss of UPR activation signal transduction, since the majority or UPR activation is transduced through IRE1 and ATF-6 signalling. To examine the role of the UPR in both normal expression and under conditions of COPI loss, MO injected and control embryos were raised to 28 hpf either in embryo water (as defined in Materials and Methods) or embryo water plus 1.8μM BFA.

Embryos injected with XBP1, co-injected with XBP1 and ATF-6 or with the control MO and then raised in blue water plus1.8μM BFA all demonstrated both a general up-regulation of BiP and COPβ' throughout the embryo as well as notochord specific maintenance at 28 hpf, demonstrating that loss of *Xbp1* or *Xbp1* and *ATF6* is insufficient to suppress activation of the UPR under conditions of COPI loss. Perhaps most interestingly though, was the observation that in untreated embryos, i.e. those raised in embryo water alone, XBP1 injected and XBP1 and ATF-6 co-injected embryos, but not control injected embryos, demonstrated a notochord specific maintenance of both BiP and COPβ'. Untreated control injected embryos demonstrated only normal expression of BiP and COPβ', with up-regulation limited to the most posterior limit of the tail (**Figure 7.9** and **Figure 7.10**).

This suggests that in MO injected embryos, which lack sufficient activation of the UPR, there is insufficient up-regulation of UPR response genes, including COPI subunits, specifically within the notochord during development. This results in a lack of ER and Golgi stress relief within the developing notochord. There is thus

continued UPR activation, since the increased secretory demand is not met, and the expression of *BiP* and *COP* subunits within the notochord is maintained.

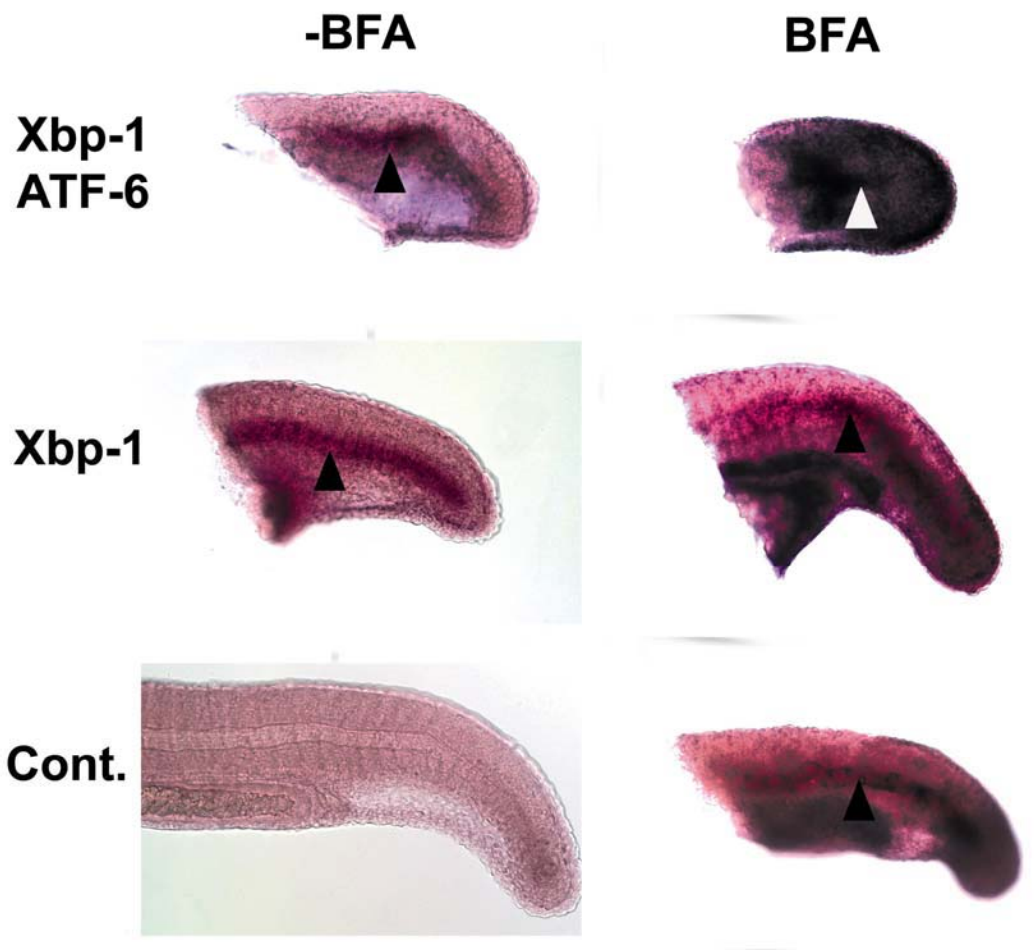


Figure 7.9 Expression of *COP*β' in normal and UPR deficient embryos.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top.
XBP-1 and ATF-6 (top row) and XBP-1 (middle row) MO injected embryos demonstrate embryos lacking full UPR activation. Under conditions of COPI loss of function (+BFA) there is maintained *COP*β' expression in UPR deficient and control embryos. Under normal conditions (-BFA), there is a lack of *COP*β' expression in control embryos. Under these condition, UPR deficient embryos show maintained expression of *COP*β' specifically within the notochord.
Arrows mark maintained expression of *COP*β' within the notochord.

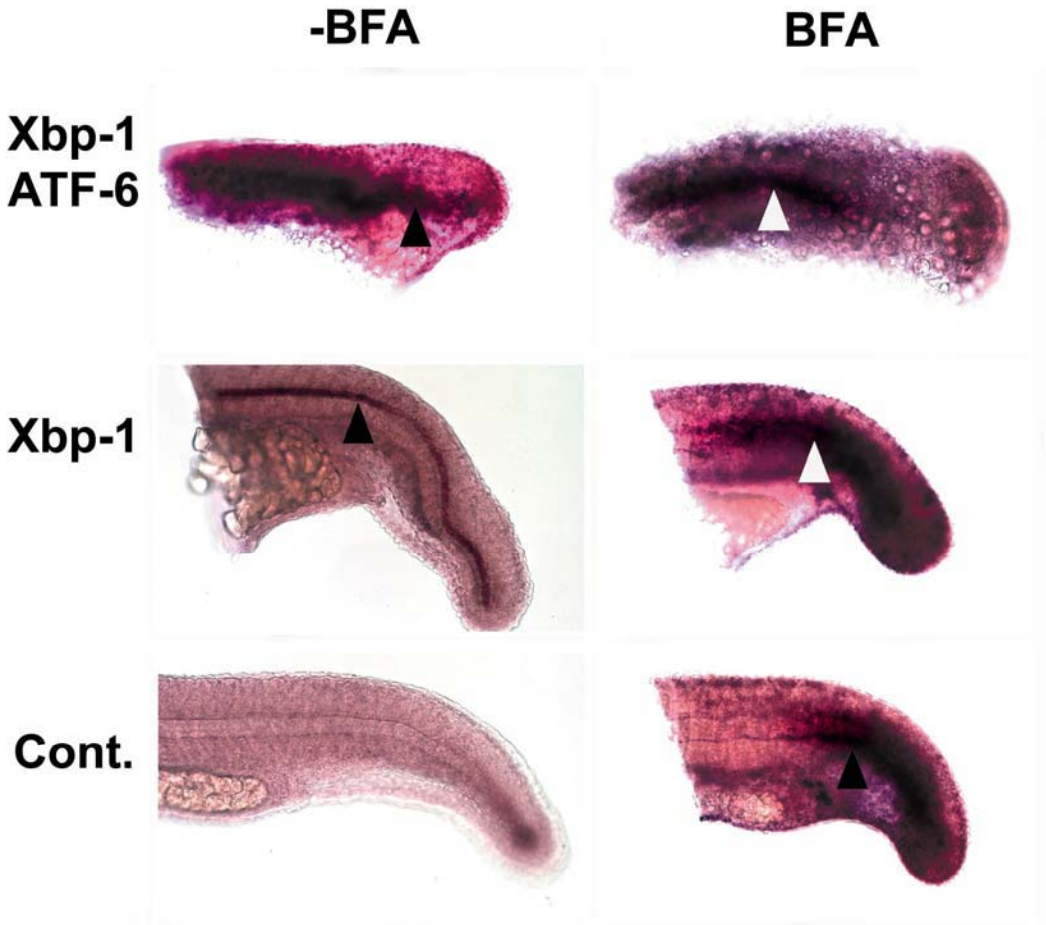


Figure 7.10 Expression of *BiP* in normal and UPR deficient embryos.

Lateral view of fixed, 24hpf tails, anterior to the left, dorsal to the top.
XBP-1 and ATF-6 (top row) and XBP-1 (middle row) MO injected embryos demonstrate embryos lacking full UPR activation. Under conditions of COPI loss of function (+BFA) there is maintained *BiP* expression in UPR deficient and control embryos. Under normal conditions (-BFA), there is a lack of *BiP* expression in control embryos. Under these condition, UPR deficient embryos show maintained expression of *BiP* specifically within the notochord.
Arrows mark maintained expression of *BiP* within the notochord.

7.7 Discussion

The expression of *ehh* within the notochord of *sny* demonstrates that there is a lack of notochord differentiation in these mutants, as observed in the *sly* mutant, which lack the zebrafish laminin γ1 gene. However, since *COP*β' is only maintained within the undifferentiated notochord of *sny* mutants at 28 hpf and is not present in the notochord of *sly* mutant embryos at 28 hpf, it can be argued that the maintenance of *COP*β', and by association the other COPI subunits that are observed within the chordamesoderm during development, is not due simply to a lack of notochord differentiation. Rather, it is the specific loss of COPI as occurs in the mutants *sny, hap* and *dop* that results in the maintenance of COPI subunit expression at 28 hpf. Reinforcing the ideal that COPI is involved in a system of auto-regulation, where conditions in which available COPI function is exceeded by demand for COPI activity lead to an up-regulation of a complete set of COPI subunits.

Loss of COPI function leads to a loss of secretory network homeostasis, since there is no retrograde transport to allow movement of secretory machinary, involved in processes such as glocosylation and protein folding, back to their correct location within the secretory network. As such, the composition of the Golgi and ER is compromised, leading to defects in post translational modification and a loss of proper protein folding, which in turn leads to a build of secretory cargo. Hence, we thought that the UPR could be engaged when COPI function is compromised and could provide a mechanism for the regulation of coatomer transcription. The expression of *BiP*, which is up-regulated under conditions of UPR activation and is commonly used as a marker for the UPR, closely resembles the expression profiles of the coatomer subunits α, β, β', ε, δ, γ2 and ζ2. Further to this *BiP* is maintained

beyond its normal temporal expression domain in the COPI mutants *sly, hap* and *dop* and in embryos treated with BFA, indicating that a loss of COPI activity leads to activation of the UPR beyond it's normal developmental expression profile. Thus, *BiP* is expressed in the same manner as the COPI notochord specific subunits, indicating that the UPR is active in the same physical and temporal domains as the notochord specific COPI subunits under the same conditions. Such observations suggest that loss of COPI function activates the UPR.

Though the UPR is active at the same time and in the same domains as the COPI subunits, it may well be that conditions where COPI activity is required and where COPI subunits are up-regulated are permissive to the activation of the UPR. Using MO knockdown techniques to either shut down the UPR response, through the removal of the signalling components ATF-6, PERK, IRE1 and Xbp1 alone and in combination, or hyperactivate the UPR, through the knockdown of BiP, the role of the UPR in development was examined through associated phenotypes.

The observed phenotype for IRE1 was considerably less severe than that noted in Xbp1 injected embryos, though they are involved in the same signalling pathway, with IRE1 acting in the intron removal dependant activation of Xbp1. The apparent difference in phenotype can be explained by the identification of only one IRE1 gene in zebrafish, where two isoforms are known in both humans and mouse but only one in *C. elegans*. Thus, the MO designed against the identified IRE1 may only knockdown one isoform, whereas knockdown of Xbp1 blocks all signalling through the IRE1 pathway. Knockdown of XBP1 resulted in defects in both the developing brain and within the notochord, with the trunk of injected embryos resembling the trunk defects observed in the COPI mutants. Embryos co-injected with Xbp1 and ATF-6 were more severe than Xbp1 alone, however they also demonstrated a lack of

proper notochord differentiation with the trunk again bearing similarities to the COPI mutants. One defining characteristic of the COPI mutants is the loss of pigment in the melanophores. In embryos co-injected with both IRE1 and ATF-6, there is an almost complete loss of pigmentation at 48 hpf. Injection of PERK MO also resulted in notochord and neural defects. Thus, removal of some elements of UPR activation, which causes a lack of UPR response, results in neural defects, a failure to differentiate notochord and a loss of pigmentation. Demonstrating a role for the UPR in, development of the brain, differentiation of the notochord and proper development of the melanophores. The latter two of these three defects are also observed in the COPI mutants, suggesting a link between the requirement for COPI and the UPR in certain developmental processes.

Knockdown of BiP results in a continual activation of the UPR system, since BiP protein acts to inhibit the activation of IRE1, ATF-6 and PERK. BiP also acts to chaperone proteins during proper protein folding within the ER. Embryos lacking BiP demonstrate amorphous and necrotic posterior trunks as well as minor neural defects when compared to UPR suppression. There is, however, some notochord differentiation in the most anterior trunk sections. BiP MO injected embryos thus demonstrate the most severe defects in the tissues that demonstrate BiP expression. This in turn suggests that BiP knockdown renders embryos more sensitive to activation of the UPR, leading to earlier activation of the apoptotic components of the UPR. Both UPR modified embryos, i.e. Xbp1, IRE1, ATF-6 and PERK injected embryos, and UPR activated embryos, i.e. BiP injected embryos, show initial establishment of dorsal-ventral and anterior-posterior axis and both demonstrate specification of chordamesoderm, though this remains undifferentiated in the UPR suppressed embryos. It therefore appears that the UPR functions after establishment

of the shield and mesendoderm, in the development of neural structures and differentiation of notochord. This is supported by the developmental profile of BiP expression, which demonstrates activation of the UPR in specific tissues initially at tailbud stage, in the chordamesoderm, and then later in both the developing brain and the differentiating notochord.

To examine the role of the UPR in regulating the expression of the COPI subunits, UPR suppressed embryos, that is, embryos injected with Xbp1 and both Xbp1 and ATF-6, were incubated with 1.8μM BFA at 18 somite stage to precipitate conditions of COPI loss of function (Coutinho et al., 2004). The expression of both *BiP* and *COP*β' in these embryos was then compared to untreated embryos at 28 hpf. In control, Xbp1 and Xbp1 and ATF-6 injected embryos, treatment with BFA resulted in the maintenance of both *COP*β' and *BiP* expression within the undifferentiated notochord and un specific up-regulation through out the embryo. Hence, removal of Xbp1 alone or Xbp1 and ATF-6 together is not sufficient to prevent activation of the UPR in response to COPI loss of function, as demonstrated by the up-regulation of *BiP* throughout the embryo and maintained within the notochord. However, this answers little about the role of the UPR in the regulation of COPI subunit expression. The observation that untreated embryos, injected with either Xbp1 or Xbp1 and ATF-6 show specific maintenance of both *COP*β' and *BiP* in the undifferentiated notochord at 28 hpf. suggests that, the suppressed UPR response in these embryos, which lack either the IRE1 signalling pathway, or the ATF-6 and IRE-1 signalling pathway, is insufficient for proper notochord differentiation. However, a lack of notochord differentiation alone does not result in the maintenance of COPI subunit or BiP expression within the notochord. Thus it can be argued that insufficient activation of the UPR during development, through

knockdown of the UPR signalling components, causes a loss of notochord differentiation and results in a lack of sufficient COPI activity. This in turn results in the expression of COPI beyond it normal temporal domain, due to a loss of COPI activity. It can therefore be argued that the UPR is not only an ER stress response, but also an essential system involved in maintaining the protein adaptory and secretory network. In this way, the UPR functions during development in tissues and cells that experience increased translatory and secretory loads as part of their normal development. In this way, the UPR is therefore suggested as a regulatory mechanism that functions in the notochord, and other tissues, during development to up-regulate many genes, including the COPI components, required to meet an increased secretory load. The UPR also acts in COPI compromised individuals, initially to maintain expression of these genes, including the COPI subunits, in an attempt to remedy abnormalities in secretion and then later in activating apoptosis in response to continued UPR activation.

The evidence presented in this chapter provides strong argument that the UPR is acting as a vital regulatory mechanism during development to maintain the ER and Golgi in cells encountering increased secretory and translatory demands. However, the evidence that the UPR is acting to regulate COPI subunit expression is less than certain. Further work, to provide more definitive evidence, therefore remains. By examining the expression of COPI subunits and *BiP* under conditions of BiP overexpression, it should be possible to more accurately defeine the role of the UPR in the expression of COPI subunits. Examination of gemonic sequence upstream of the COPI subunits may reveal common regulatory elements. Such elements could then be compared to characterised UPRE sequences and then examined through the attachment of marker genes to such regulatory elements. Despite the work still to

perform, the demonstration that the UPR is active when and where COPI subunits are upregulated and that a lack of UPR activation results in similar developmental defects to those observed in COPI mutants strongly links the UPR to COPI subunit expression. Combined with the demonstration that a lack of proper UPR activation leads to both maintained UPR activation and COPI subunit expression, the evidence provides strong, though incomplete, support for a model where the UPR is functioning during the development, including within the notochord, to up-regulate genes, including the COPI subunits, involved in secretion and post-translational modification.

7.8 Summary

- Maintenance of COPI subunits in COPI mutants is not due to a lack of notochord differentiation, but a specific loss of COPI function.
- *BiP* mRNA has the same expression profile as the notochord specific COPI subunits during development and in the COPI mutants.
- Knockdown of the UPR results in comparable notochord and melanophore phenotypes to *sny, hap* and *dop*.
- Over-activation of the UPR results in apoptosis in the trunk and head.
- Knockdown of XBP1 or XBP1 and ATF-6 is insufficient to prevent COPI maintenance and prolonged UPR activation in response to COPI loss of function.
- Knockdown of XBP1 or XBP1 and ATF-6 results in COPI maintenance and prolonged UPR activation in wild type 28 hpf embryos.

- The UPR may be involved in both the normal developmental expression profile of COPI subunits and abnormal maintenance in COPI mutants.
- The UPR plays an essential role in development.

Chapter Eight

Discussion

8 Discussion

In recent years many families of genes have been identified to have vital roles in development. Many transcription factors have been identified as essential for proper development, acting in different cells to specify distinct fates. These transcription factors include the *Hox* gene family, which act in patterning both positional and temporal identity, the helix-loop-helix factors MyoD and Neurogenin, which act in specifying cells to a muscle or neural fate respectively and the t-box family, which includes the gene no-tail, which was identified as having an essential role in notochord development (Ma et al., 1996; Schulte-Merker et al., 1994; Weintraub et al., 1991; Weintraub et al., 1989). Further factors include secreted proteins, which act in signalling between developing cells and include the Wnt's, FGF's and the TGFβ family. More recently, a range of other factors have been identified as having essential roles in development. Work on the zebrafish notochord mutants generated as part of the 1996 ENU mutagenesis screen, has demonstrated a role for both the extracellular matrix and secretory maintenance in development. The mutants *gup, sly* and *bal* lack the laminin subunits β1, γ1 and α1 respectively and show defects in both neural and notochord development (Parsons et al., 2002b; Pollard, 2002). The mutants *sny, hap* and *dop*, which encode COPα, COPβ and COPβ' respectively, have demonstrated a role for secretory maintenance in development of both the notochord and the melanophores ((Coutinho et al., 2004) This thesis). Though much is now known concerning development, precisely how the range of developmentally essential factors interact to bring about the differentiation of cells and the generation of complete tissues is not well understood.

Much work, in a range of model organisms and developmental systems, is currently being performed to answer many of the un-resolved questions in developmental biology.

As part of this thesis, I have investigated the process involved in early vertebrate development, using the zebrafish notochord as a developmental system. Through the use of both characterised and un-characterised notochord mutants, I have aimed to further define what genes and systems are important in the process of notochord differentiation and to expand this understanding into the greater scheme of development. I have characterised the gene responsible for the notochord specific mutant *doc* and established that it encodes a novel and highly conserved 14 WD40 domain protein. I have also characterised the *hap* and *dop* loci, confirming that they encode the COPI subunits COPβ and COPβ' respectively. Continuing this, I have characterised the other COPI subunits and, through analysis of COPI expression and regulation, I have implicated the UPR as an essential regulatory system involved in development.

8.1 A Novel Gene with a Role in Zebrafish Notochord Development

8.1.1 Cloning the Zebrafish Notochord Mutant *doc*

By analysing the inheritance of polymorphic markers in *doc* mutant embryos with a systematic screen of SSLP markers, the mutant locus was defined to chromosome 18. The marker Z9484 demonstrated linkage to the *doc* locus and through analysis of known linked markers, the mutation was defined to a region

between the markers Z9484 and Z7417. Using publicly available genome sequence, these markers were placed on the genetic map, defining a region of ~12Mb between 5.5Mb and 18.2Mb. Through selection of identified INDEL's and BAC ends within this region, markers of ~200bp were designed and then examined for polymorphism. Polymorphic markers then assisted in fine mapping the *doc* locus to a region of ~0.5Mb between a BAC end marker at ~11.1Mb and an INDEL marker at ~11.5Mb. Examination of genomic sequence identified, three candidate genes, with homologies to Syntaxin-8, Cadherin-13 and a novel, conserved protein displaying multiple WD40 domains.

So, by examining the frequency of meiotic recombination, the *doc* locus was defined to a region of ~0.5Mb, where three candidate genes were identified. This approach was assisted by known and previously mapped SSLP markers, and further refined through the use of genome sequence to generate further markers. Genome sequence then allowed the identification of appropriate candidate genes, which could then be tested through antisense MO knock-down and whole mount *insitu* hybridisation.

8.1.2 A Novel Multiple WD40 Domain Protein Encodes *doc*

Using MO antisense knock-down, the roles of the most likely candidate genes within the region defined for *doc* were examined. In this way, a novel protein containing multiple WD40 domains was determined to have an essential role in notochord development. Antisense MO knock-down of the mRNA encoding this protein results in a lack of AP extension, formation of 'U' shaped somites and a loss of proper notochord differentiation. Staged expression analysis of this mRNA

demonstrated up-regulation specifically within the notochord at 18 somite stage and a lack of notochord expression at 6 somite and 24 hpf. Hence, expression of *doc* is up-regulated in the notochord between ~11 hpf and 24 hpf and is thus expressed at stages before the notochord is fully differentiated and before early markers are extinguished. Expression of *ehh* in doc MO injected embryos has demonstrated that there is a lack of proper notochord differentiation in knock-down embryos. From this supporting evidence, it is suggested that the phenotype in *doc* mutants results from mutations in the gene encoding the novel, conserved WD40 domain protein. However, definitive proof that this gene does indeed comprise the *doc* locus remains to generated. Sequencing the precise mutation from both cDNA and genomic DNA would provide final confirmation that this gene is indeed the *doc* locus.

The precise function of this gene has yet to be determined and the novel nature of the gene has meant that no function can be derived from information on studied homologues. However, the multiple WD40 domains suggest that doc is interacting directly with other proteins in the process of notochord development. Though precisely what doc is interacting with remains to be established, it may be that it acts in establishing a link between the notochord BM sheath and the notochord itself. The multiple WD40 domains suggest that doc may be function via direct interections with other proteins. As such, doc may coordinate the cytoskeleton in response to basement membrane formation, or might stabalise a signal required for vacuolation that is received in response to BM formation. Additionally, the multiple WD40 domain may enable interactions with secretory components, for examble the COP subunits, which also demonstrate WD40 domains. So it may be that doc acts as a link between secretory maintanence and notochord vacuolation. A role for doc in signalling between notochord differentiaion and BM formation fits with observations

concerning general notochord differentiation, where a lack of sheath in *X. laevis* has been noted to result in a failure of notochord vacuolation and differentiation (Adams et al., 1990). Considering the nature of notochord development, where cells vacuolate and press against the surrounding sheath at high pressure to strengthen the notochord through turgor pressure, it seems reasonable to assume that a system of signalling to ensure proper sheath formation would be in place, since vacuolation prior to sheath formation would be fatal. It may well be that doc functions in just such a pathway, acting in some way to signal the completion of the BM sheath to the notochord cell. Expression analysis and MO knock-down analysis places just such a role firmly within the realm of doc action.

The fact that the doc remains highly conserved throughout evolution suggests that it has a vital function. A lack of functional information in other species has left many questions remaining concerning its function in zebrafish development, but also places the mutant *doc* in a position of considerable interest. Establishing the role of doc in the development of the zebrafish notochord would help define the role of this gene in other organisms and may well highlight a conserved mechanism of development throughout evolution. Thus, continued study of *doc* is of great interest.

8.2 The Role and Regulation of the Secretory Network in Development

8.2.1 A Role for COPI in Development

Work on the zebrafish notochord mutant *sny*, identified the gene COPα, a member of the seven subunit COPI coat complex, as essential for both notochord

development and pigmentation formation in melanophores (Coutinho, 2001). The phenotypic similarity between the mutants *sny, hap* and *dop* suggested that *hap* and *dop* may encode other COPI subunits, a suggestion that was reinforced by the ability of MOs against COPβ and COPβ' to phenocopy *hap* and *dop* respectively.

As part of this thesis, mutations were identified in COPβ and COPβ' in *hap* and *dop* embryos respectively. Sequencing of cDNA from *hap*^{tm285b} mutant embryos identified a substitution within COPβ that encodes a truncated protein of 499 amino acids. Sequencing of cDNA from *dop*^{m341} embryos identified a substitution within COPβ' that encodes a truncated protein of 761 amino acids. Thus, zygotic COPβ and COPβ' in *hap* and *dop* embryos is non functional and, since COPβ and COPβ' are both essential for the formation of the fully functional seven subunit COPI complex, there is a lack of zygotic COPI activity. Examination of COPβ and COPβ' mRNA expression during normal development demonstrated that both subunits are supplied maternally and expressed ubiquitously at early stages. These subunits then become specifically up-regulated in the chordamesoderm/notochord and neural structures from tailbud stage onwards. Not only did this expression match that previously observed for COPα, but it also matched that observed in the COPI subunits δ, ε, γ2 and ζ2.

Initial identification of *sny, hap* and *dop* as the COPI subunits α, β and β' respectively, was somewhat unexpected, since the COPI complex is an essential housekeeping complex, required to maintain the structure and composition of the secretory networks and considering that removal of COPI function in yeast is lethal. Thus, characterising these COPI subunits, and hence COPI function, as responsible for specific developmental defects was unexpected as removal of such a ubiquitously expressed gene complex would be thought to be lethal. However, in reconsiliation of

the essential nature of coatomer function, embryos do die by widespread necrosis by 48 hpf. By applying current understanding of notochord developmental processes, the specific phenotype observed can be readily understood. COPI function is provided maternally, since it is essential to cell survival and zygotic transcription does not initiate until the MBT. This maternal contribution of functional COPI is sufficient for the initial survival of the embryo, even when the zygotic contribution is absent. The half-life of the COPI complex in a mammalian cell lines has been measured to be 28 hours (Lowe and Kreis, 1996). However, the notochord, which is one of the first fully differentiated tissues in the zebrafish, experiences an increased demand for secretion. Notochord cells must assist in the formation and construction of the notochord basement membrane, of which laminins are a major component (Parsons et al., 2002b; Pollard, 2002), and must also undergo vacuolation. Both of these processes place increased demands on the secretory network. Thus, since COPI function is required for maintenance of the secretory pathway, a lack of COPI function will have a more dramatic effect on notochord development. As such, as the notochord differentiates, it out-strips the maternal complement of COPI due to its increased secretory requirements. The developing chordamesoderm/notochord must therefore support the maternal COPI supply through zygotic translation, which is supplied by the notochord specific expression of the COPI subunits between tailbud and 18 somite stages. However, in the mutants *sny, hap* and *dop*, the COPα, COPβ or COPβ' subunit is defective, respectively, and hence zygotic COPI function is compromised. This then results in the specific phenotype observed before general necrosis. This same reasoning can be applied to the observed defect in melanophore pigment development, since development of proper pigmentation requires the secretory network and thus places increased demands on COPI activity. Thus,

though COPI activity is required ubiquitously for cell survival, mutations in individual COPI subunits results in specific developmental abnormalities in specific cells and tissues that place an increased demand on the secretory network, early in embryogeneis, that exceeds the maternally contributed COPI activity. Later, loss of zygotic COPI activity results in wide spread cell death due to loss and dilution of the maternally provided, functional, COPI complex.

Previous analysis of *COPα* expression had demonstrated that mRNA is specifically up-regulated within the developing notochord from tailbud stage until approximately 18 somite stage, with the notochord specific expression becoming extinguished in all but the most posterior developing tip of the notochord by 24 hpf. Analysis of *COP*β and *COP*β' mRNA expression demonstrated a similar pattern of expression, which is befitting of the notochord defect in these COPI mutants. Perhaps most interesting though, was the observation that *COPα* expression was abnormally maintained in all three COPI mutants and in embryos treated with BFA, a general inhibitor of COPI function (Coutinho, 2001). This maintenance of subunit expression within the notochord of COPI mutants at 28 hpf was also demonstrated for *COP*β and *COP*β'. Though many early notochord markers, including *ehh*, are maintained in undifferentiated notochord, due specifically to the lack of differentiation, the COPI subunits are not. Where notochord expression of *ehh* is maintained in both *sly* mutants, which lack laminin α1, and *sny* mutants, which lack COPα, COPα is maintained only in the notochord of *sny* mutants at 28 hpf. Thus, it is a specific loss of zygotic COPI function that leads to the maintenance of subunit expression at 28 hpf. This observation, combined with the observed phenotype and expression of COPI subunit mRNA during normal development, suggested a

mechanism of regulation where COPI activity acts in some way to regulate expression of the COPI subunits.

Such a system would fit with observations of the COPI mutants. Under normal conditions, in wild type embryos, the maternal contribution of COPI activity is sufficient to ensure cell and embryo survival until zygotic transcription can begin. It is also sufficient for survival in the majority of cells and tissues in the developing embryos until approximately 48 hpf, when embryos lacking zygotic COPI function undergo widespread cell death. However, in the notochord and melanophores, where there is an early increased demand for secretory network activity, to act in producing the basement membrane sheath and pigmentation respectively, the maternal contribution is insufficient. In these cells, the requirement for COPI exceeds the level of available activity. Under such conditions, the loss of sufficient COPI activity acts to up-regulate expression of COPI subunits, causing the observed notochord specific expression of COPI subunits at tailbud to 18 somite stage. The loss of zygotic COPI subunits in the mutants *sny, hap* and *dop* results in an inability to supplement the maternal COPI activity and thus, the tissues in which the maternal compliment of COPI is insufficient are affected before widespread cell death is apparent. However, since these embryos are unable to provide additional zygotic COPI activity, the increased demand on the secretory network is maintained as is the requirement for COPI activity, thus, the expression of the COPI subunits within the notochord is maintained beyond its normal temporal profile.

In such a system, available COPI can be viewed as a pool of activity, initially supplied maternally. This maternal pool is sufficient for cell maintenance throughout the early embryo, with the exception of the notochord and the melanophores, where demand for secretion exceeds the activity available in the maternal COPI pool.

Under these conditions of loss of available activity, where all COPI is recruited to the highly active secretory network, the free activity available in the pool is insufficient. This signalling results in the the up-regulation of COPI subunits to increase the pool of zygotic COPI activity. This restores the free activity of COPI to a level that is sufficient so that additional COPI is no longer required and subunit expression is extinguished. So, in wild type embryos, by tailbud stage, secretory network activity is sufficiently high to exceed the pool of maternal COPI and cause the up-regulation of COPI subunits, but by 24 hpf, zygotic COPI transcription has re-established the pool of free COPI activity and therefore COPI subunit expression is extinguished in the notochord. However, in the COPI mutants, the zygotic COPI is non-functional and hence subunit expression within the notochord is not extinguished, as the pool of free COPI activity cannot be supplemented. Thus, COPI is acting in an auto-regulatory feedback loop to maintain sufficient activity during development.

Examination of the remaining *six* coatomer subunits demonstrated that the subunit ε, δ, γ2 and ζ2 are also up-regulated specifically within the notochord and maintained in the notochords of COPI deficient embryos, thus demonstrating that, like *COPα*, *COP*β and *COP*β', these subunits are up-regulated under conditions of depleted free COPI activity. However, the subunit COPζ is not. So, under conditions where the pool of available COPI activity is depleted, a complete set of seven COPI subunits is up-regulated, via an auto-regulatory feedback mechanism, to restore the level of COPI activity.

8.2.2 The Effect of Loss of COPI Function

Visualisation of Golgi and ER structure in living cells, using fluorescent tagged proteins enabled the effect of COPI loss of function to be examined through knock-down of the COPα subunit with an ATG targeted MO or general inhibition with BFA. Loss of COPI function most prominently results in a complete loss of vacuolation within notochord cells. Wild type notochord cells demonstrate clear and distinct Golgi and ER structure, though this is confined to a small region of the cell as a result of vacuolation. In contrast, the notochord cells of COPα MO injected embryos have a complete lack of vacuolation, as demonstrated by their vastly diminished size and the observation that both the Golgi and the ER are spread throughout the cell. This dispersal of the ER and Golgi was also observed in the muscle cells of COPα MO injected embryos. In both morphant notochord and muscle cells, there is a breakdown and dispersal of the ER and a fusion and dispersal of the Golgi, due to the loss of retrograde transport. In wild type notochord and muscle cells, the ER and Golgi are distinctly localised around the nucleus and organised into discrete organelles. Thus, COPI function is vital to maintain the proper structure of both the Golgi and the ER, by acting to transport "lost" components back to their proper organelle position.

By examining the localisation of the fluorescent marker proteins over time, the nature of Golgi and ER breakdown was observed and, additionally, the time scale of this dispersal was identified. At 28 hpf, morphant cells closely resemble those observed in wild type embryos. By 31 hpf however, the ER has become completely dissembled and its contents are dispersed throughout the cells, with no distinct localisation. This breakdown and dispersal can be observed to occur gradually between 28 hpf and 31.hpf, demonstrating that the lack of COPI function becomes critical to ER structure at this stage. Golgi structure is also grossly amorphous by 31

hpf, showing a fusion of individual organelles and a dispersal of Golgi contents throughout the cell. Thus, it appears that the loss of Golgi and ER structure is not apparent at 28 hpf, but develops from this time causing the breakdown of the ER, fusion of Golgi organelles and the dispersal of secretory network contents throughout the cytoplasm.

Beginning at 28 hpf, the Golgi and ER loose normal structure and disperse throughout the cell rapidly, becoming grossly malformed over a period of two hours. This loss of Golgi and ER structure occurs in cells throughout the developing embryos. Defects in notochord differentiation are obvious by 32 hpf, COPα morphant notochord cells are considerably smaller than their wild type counter parts. Morphant notochord cells show a complete lack of vacuolation and the ER and Golgi are dispersed throughout the cytosol.

8.2.3 The UPR as an Essential Regulatory Mechanism in Developmental

The UPR is an important and only partially understood response mechanism to ER stress. Under conditions of increased secretory or protein folding demand, the UPR is active and is responsible for up-regulating many genes involved in post-translational modification, protein folding and protein transport, which then act in the ER and Golgi to alleviate stress. Much of the current understanding of UPR activation and activity stems from work in yeast and recent analysis in *S. cerevisiae* has demonstrated that amongst the many thousands of genes up-regulated in response to UPR activation, are several COPI subunits (Travers et al., 2000). This observation, combined with the nature of COPI and the observed auto-regulatory

expression of the COPI subunits, suggested that the UPR may be responsible for regulating coatomer expression during development.

The expression profile of *BiP*, a major regulator of the UPR and a commonly used marker of UPR activation, is highly similar to that observed for all seven of the notochord specific COPI subunits. Thus, it appears that the UPR is active at the same time and in the same tissues that there is specific up-regulation of COPI subunits. This observation, along with the observation in yeast that activation of the UPR result in up-regulation of COPI subunits, reinforced the suggestion that the UPR is acting to regulate the expression of COPI. Given the nature of notochord differentiation, this makes considerable sense. During notochord differentiation there is a requirement for basement membrane sheath formation, which can be provided by both surrounding tissues and by the notochord itself (Coutinho et al., 2004; Parsons et al., 2002b). The formation of this basement membrane requires the secretion of proteins, such as laminin, from notochord cells. The demand for these proteins places an increased demand on the ER's protein folding capacity and on the ER's and Golgi's ability to properly secrete proteins. This increased demand would, in cells with only the basic, maternal complement of protein folding and secretory compounds, result in a build of unfolded proteins within the ER. Thus, the UPR would be activated and the necessary components to relieve this unfolded protein backlog would be up-regulated. It therefore seems, considering the observation that UPR is active within the notochord during development, maintained within the notochord of COPI deficient embryos and that the UPR results in expression of COPI components in yeast, that the UPR is acting during development as an essential regulatory mechanism to meet specific demands in cells and tissues, in response to

increased secretory and protein folding demands, to maintain the structure and composition of both the Golgi and ER.

To further define the role of the UPR during development, MO's were targeted against the ATG start of translation sites of the key signalling components in the UPR: IRE1/XBP1, ATF-6, PERK, and the key regulator of UPR activation, BiP. By knocking down the signalling components of the UPR, the ability of the UPR to activate in response to unfolded protein build up is compromised. As such, the level of UPR response is severely reduced. In such embryos, there is a distinct lack of notochord differentiation. The observed defect in notochord differentiation in embryos deficient in UPR activation closely resembles the notochord differentiation defect observed in the COPI mutants *sny, hap* and *dop*. Additionally, embryos raised to 48 hpf demonstrate an obvious similarity to the COPI mutants, not only in terms of lack of notochord differentiation and associated defects, but also in the distinct lack of proper melanophore development.

Thus, a lack of UPR activation appears to phenocopy the COPI mutants in terms of the lack of notochord differentiation and loss of pigmentation, supporting a role for the UPR in the requirement for COPI in both notochord and melanophore development. The UPR is thus acting in response to increased secretory and post-translational adaptory demands by up-regulating the proteins involved in such processes, including the COPI subunits. Interestingly, knockdown of UPR activation also results in obvious neural defects. This fits with the observed expression profile of BiP, where expression is noted not only in the developing notochord, but also in the developing brain. Interestingly, though COPI subunits are also expressed with the developing brain, the COPI mutants show no significant defects in neural development. However, it may be that the UPR is acting in both the notochord and

the developing brain in response to differing pressures on the ER as a result of differentiation and that this activation results in the up-regulation of all UPR target genes, including the COPI subunits. However, only a subset of these genes are necessary to alleviate the specific stress of each developing tissue. Thus, in notochord, the UPR up-regulates all the UPR genes, where only a subset of genes, including COPI, are required for proper notochord differentiation, whereas in the brain, a different subset, not including COPI, is necessary for proper development. Hence the notochord specific defect in COPI mutant embryos and the combined notochord and neural defects in UPR inactivated embryos.

Further evidence for the UPR's role in notochord development has been resolved from expression analysis in UPR inactivated morphant embryos. In such embryos it was clearly demonstrated that UPR inactivated morphant's do not have a completely inactivate UPR, despite the obvious notochord, melanophore and neural defects, since BiP and COPβ' were both up-regulated in response to BFA treatment. Indicating that a partially active UPR is insufficient to maintain proper notochord development. However, more informatively, the partially UPR inactivated embryos demonstrated a distinct maintenance of *COP*β' and *BiP* at 28 hpf when raised under normal conditions, similar to the COPI mutants. In such embryos, the partially inactivated UPR is insufficient for the required gene up-regulation necessary to meet the increased demands for Golgi and ER activity, resulting in the observed defects in development. This also results in maintained activation of the UPR, since the stress on the ER and Golgi is not alleviated by zygotic transcription. This maintained expression of *COP*β' and *BiP* at 28 hpf closely resembles the maintained expression of *COP*β' and *BiP* in COPI mutants. In both the COPI mutants and UPR activation morphant's, there is a lack of components necessary to alleviate the increased

demands on the Golgi and ER, due to either a specific lack of functional COPI or the lack of many important genes, including the COPI subunits, respectively, and as such, both result in maintained activation of the UPR and therefore the maintained expression of *COP*β' and *BiP* at 28 hpf.

It thus appears that the UPR is active during development in tissues and cells that encounter increased translational and secretory demands, to help maintain the structure, composition and function of the ER and Golgi. The UPR is active in both the notochord and brain during development, and a loss of proper activation causes defects in the development of neural structures, notochord and melanophores as well as resulting in widespread necrosis by 48 hpf. However, the specific stresses of these tissues vary, such that loss of COPI function, which is up-regulated by the UPR, results only in defects in notochord and melanophore development. So, the UPR responds to general ER stress during development, to up-regulate the range of gene regulated by UPR activation, though only a subset are required for alleviating the specific stresses within the developing tissue or cell. Further work to demonstrate decisively that the UPR does indeed act to regulate COPI expression during development remains, and is a source of continued effort. However, the evidence put forward in this thesis supports the idea that the UPR is required for proper development and that this requirement may result in the upregulation of UPR target genes involved in membrane trafficking, secretion, ER and Golgi maintenance, translation and post-translational modification, which would include the COPI subunits and numerous other genes involved in vesicular transport.

Such a role for the UPR is further supported by recent work in plasma cell differentiation (Brewer and Hendershot, 2005; Iwakoshi et al., 2003a). In such work, it has been demonstrated that XBP1 is essential for differentiation of plasma cells

(Iwakoshi et al., 2003b). This work demonstrated that IRE1 spliced XBP1 was able to restore immunoglobulin production production in $XBP1^{-/-}$ cells, where unspliced XBP1 was unable to do so and that XBP1 splicing occurs normally during terminal B cell differentiation, linking the UPR to differentiation of plasma cells. This, coupled with the observation that XBP1 splicing is dependent on the production of immunoglobulin, since the prevention of protein synthesis by cycloheximide in B cells results in a loss of XBP1 splicing (Iwakoshi et al., 2003b), links the UPR with the differentiation of plasma cells via its role in ER stress and in maintaining the secretory and translatory potential of a cell. Indeed, when viewing plasma cells as antibody factories', which are responsible for secreting massive quantities of soluble protein, a role for the UPR in their development can be compared directly to the role proposed for the UPR in notochord, as put forward in this thesis.

The demonstration that the UPR, which is considered a stress response mechanism, acting to protect and restore the function of the ER under conditions unfolded protein stress, acts during development to meet the specific secretory and translatory demands of developing cells demonstrates that cell stress protection mechanisms can and do function specifically during development. Such an observation opens up the possibility that other "stress" response mechanism might also have vital roles during development to maintain cells under the strenuous conditions they encounter as they differentiate.

8.3 Future Work

The notochord has proven to be a useful developmental system in zebrafish. Work on the ENU generated zebrafish notochord mutants has demonstrated essential

and specific roles in early development for both Laminin and Coatomer, despite their roles throughout the embryo. Such roles would have been difficult to define using mouse, where loss of laminin can lead to implantation defects and where loss of coatomer function would lead to early lethality. This thesis has concentrated on two related projects; the positional cloning and identification of the last zebrafish notochord mutant, *doc* and the characterisation of COPI expression and regulation during development. Though many questions still remain, especially concerning the nature of doc's role in notochord differentiation and its possible signalling function downstream of sheath formation, much has been learnt about the process of both notochord development and the general mechanisms of development.

Obvious immediate aims are the further characterisation of the role of doc in notochord development. One of the most beneficial examinations would be to determine what other proteins interact with doc, since doc may be acting directly in signalling the notochord cell to differentiate in response to sheath formation and thus, may be interacting with either the basement membrane sheath directly or with transmembrane proteins that are themselves interacting with the sheath. Expression of a tagged protein would enable isolation of doc from embryos, and associated proteins could rapidly be examined through mass spectroscopy techniques. Associated binding partners could also be easily identified through the use of yeast two hybrid screens, of which there are several commercially available for the zebrafish. It should therefore be relatively simple to identify proteins that are able to interact with doc and from this information, it should hopefully be possible to place doc into a system or signalling mechanism that befits its role in notochord differentiation. It may be that such information will enable the linking of doc to the differentiation and vacuolation of notochord cells and to the formation of the

basement membrane sheath, or it may be that doc is involved in other processes required for differentiation. The role of any identified interactors could be relatively easily tested using a MO knockdown approach and by identifying proteins that interact with doc it should hopefully be possible to place this novel gene into a system involving known and characterised factors, thus helping to reveal the mechanism by which doc functions in notochord differentiation. Further to this, information about how this protein functions should also be revealed by its structure and so another obvious avenue for investigation is the crystallisation of the protein and examination of its three-dimensional structure. By comparing this information to the known structures of well characterised proteins, it should be possible to hypothesise how doc functions.

The identification of *doc* represents the cloning of the last of the seven "dwarf" mutants in zebrafish. These mutants have revealed specific roles for the laminin chains α1, β1 and γ1, as well the coatomer coat protein, in early development. Both of these families of mutants represent well-characterised proteins that can be attributed clear roles in the development of the notochord, where laminin forms a vital part of the notochords basement membrane sheath and where COPI acts in both secreting the components of the sheath and in vacuolation. The identification of *doc* and the further characterisation of how it is acting during development to maintain notochord differentiation should hopefully provide a link between the formation of the sheath, vacuolation and proper differentiation, offering the opportunity to identify further processes and components that act during the development of the notochord.

The mutant *sly* was demonstrated to encode a non-functioning COPα protein (Coutinho, 2001) and MO knockdown of the subunits *COP*β and *COP*β' was

demonstrated to phenocopy the mutant *hap* and *dop*. Work as part of this thesis demonstrated that the mutants *hap* and *dop* encode non functioning COPβ and COPβ' subunits respectively. Analysis of the COPI mutants has revealed that the majority of COPI subunits are up-regulated within the notochord during development and maintained in the notochords of COPI mutants. Examination of the mechanism of this regulation demonstrated that the UPR stress response mechanism acts as an essential regulatory mechanism during development to maintain the function and composition of the ER and Golgi. The UPR is active in the notochord during development and acts to up-regulate genes essential for differentiation, including the COPI subunits. However, this work also revealed the essential nature of the UPR for proper neural development, where a lack COPI function does not appear to result in obvious neural defects. Thus, examination of which UPR regulated genes are required for proper neural development is an obvious extention to current work. It may be that the UPR is functioning in several developmental process, including later processes that have not been revealed through MO knockdown and that UPR regulated genes can be divided into different classes depending on which developmental process/processes they are required for. For example, though the majority of the COPI subunits were up-regulated in all tissues in response to UPR activation, one subunit demonstrated discrete expression patterns where mRNA levels were raised only in specific regions of UPR activation. Thus, it may be that the UPR has several specific responses, comprising varying sets of response genes, as well as a unanimous gene set, up-regulated under any conditions of UPR activation. Examination of what genes are up-regulated in either neural or notochord tissue during development, through, for examble, micro-array analysis, would help to define if the UPR is able to function in such a manner and if so, then what genes are

regulated under what conditions. On a more general note, identifying what genes respond to UPR activation would also be of interest. Since many UPR responsive genes have been classified in yeast, it should be possible to identify homologues for many of these in zebrafish from the genomic sequence and then characterise if they respond to the UPR in zebrafish as they do in yeast. It would then be relatively simple to identify upstream genomic sequences for these genes, which could then be used to perform comparative searches for conserved regulatory elements. Such searches have demonstrated success in yeast (Patil et al., 2004). These regulatory elements could then be used to search for further UPR regulated genes.

Much of the emphasis of developmental biology has been on the events involved in altering both the genetic and physiological make-up of cells during development of a properly differentiated and patterned adult. However, the mechanisms involved with maintaining the basic functions of the cell during these complex events have been little considered. Here it has been shown that the UPR, which is involved with maintaining the function and composition of the ER and Golgi under conditions of increased secretory and translatory load, acts during development to maintain the development of cells that exceed the natural complement of Golgi and ER functional proteins. Moving beyond the specific scope of the UPR, the demonstration that such a protective mechanism is required for proper development opens up the possibility that other such mechanisms could also be functioning in such a manner. Work on the heat shock response in *Arabidopsis* has demonstrated a chaperone function for heat shock proteins under normal conditions, allowing aberrant proteins to fold correctly and acting as a "buffer" for mutations (Queitsch et al., 2002). Thus, it may be that the heat shock response functions in a similar way during zebrafish development, allowing proteins to fold

correctly under the stressful conditions encountered during development. In a broader view, it is possible to see how such stress response mechanisms might evolve in simple organism to allow survival in non-optimal conditions but, as they evolve into complex muticellular organisms, such mechanisms become vital for cell survival during the strenuous conditions encountered during differentiation. Such mechanisms would provide protection against potentially lethal conditions that would arise as the processes of development evolved and may well be essential to provide room for the 'evolutionary experimentation' that occurred during the transition from single celled eukaryotes to a complex, highly patterned, muticellular organisms.

Analysis of the UPR and other "stress" response mechanisms may yield much information about how a developing embryo is protected and buffered against the stresses encountered during normal development and may provide much information about the regulation of the essential maintenance processes involved in the complex events that occur during the development from a simple unspecified collection of cells to a fully functioning adult.

8.4 Summary

- *doc* encodes a novel and conserved 14 WD40 domain protein.
- The mutants *hap* and *dop* encode COPβ and COPβ' respectively.
- All of the COPI subunits, with the exception of COPζ, are expressed in the notochord during development
- The same subunits are maintained in COPI deficient embryos.
- Loss of COPI results in a breakdown of the ER and Golgi.

- The UPR is vital for proper notochord, melanophore and neural development.
- The UPR may be responsible for up-regulting COPI subunits under conditions of ER stress to maintain Golgi and ER function.

References and Appendices

References and Appendices

Appendices

COPb2

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COPb1

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COPe

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COPg2

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COPd

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IRE1

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ATF-6

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PERK

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XBP1(inactive)

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XBP1(active)

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BiP

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