3 Library construction

3.1 Introduction

The aim of my project is to generate a panel of homozygous mutations for a recessive genetic screen. As I described in the first chapter, there are several different mutagenesis methods to create mutations in ES cells, and each of them has their own advantages and disadvantages. The bottleneck for all the gene-targeting based methods is that they require the labour-intensive and time-consuming generation of the targeting vectors as well as genotyping the targeting events. For all the random mutagenesis methods, although they can mutate the first allele of a gene efficiently, it is hard to disrupt the second allele and make the mutation homozygous. So a new method was needed to make the generation of homozygous mutations high-throughput, so that more focus can be put on the design of sophisticated functional genetic screens.

The original design of my project was: 1) create a cell line for induced mitotic recombination on chromosome 11; 2) target some important developmental genes in this cell line by homologous recombination; 3) induce mitotic recombination by transient expression of Cre to generate homozygous mutant ES cell clones; 4) use ES cell *in vitro* differentiation to study the function of these genes. Though this strategy still requires the genotyping of each locus that has been disrupted, it effectively saves one step of targeting and genotyping. Since I chose to use *E. coli* recombination to make gene-targeting constructs, which allows longer homology arms and has less chance of introducing point mutations, it is possible to achieve high targeting efficiency (Copeland, Jenkins et al. 2001; Liu, Jenkins et al. 2003). By calculation, if the targeting efficiency of a construct is 10%, then half of these (5%) will happen on the same chromosome as the 3' *Hprt* cassette, and 60% of the HAT resistant clones (3%) will carry homozygous mutations (Liu, Jenkins et al. 2002). So in principle, it is possible to omit the genotyping of the first targeting event, pool the transformants and induce mitotic recombination directly. The homozygous clones can be efficiently recovered from the HAT resistant clones.

During the process of making the starting cell line for induced mitotic recombination, Meredith Wentland, a former PhD student in Dr. Allan Bradley's lab, successfully isolated gene-trap insertions in a chromosome-specific way using a method called "regional trapping". Given this observation, we decided to combine the two methods (induced mitotic recombination and regional trapping) to generate random homozygous gene-trap mutations in a chromosome-specific way and thus completely save the trouble of constructing different targeting vectors and designing genotyping strategies for targeting of each different locus.

Since both studies have been carried out on mouse chromosome 11 (Liu, Jenkins et al. 2002), all the targeting vectors are readily available and the efficiencies of induced mitotic recombination and regional trapping on chromosome 11 are already known, we decided to perform this experiment still on chromosome 11. The design of my project was changed accordingly to: 1) create a cell line for induced mitotic recombination on chromosome 11; 2) perform random mutagenesis in this cell line by gene-trapping; 3) induce inversion by transient expression of Cre to select for trapping events on chromosome 11; 4) induce mitotic recombination by transient expression of Cre to generate homozygous mutant ES cell clones; 5) screen these ES cell clones by an *in vitro* differentiation assay to study the function of trapped genes.

Some modifications of Liu and Wentland's original experiment design needed to be made to suit my purpose. For example, both experiments use Cre/*lox*P system, and it is necessary to use different *lox* sites for the two steps so that they will not interfere with each other. There are also considerations about the trapping strategy: 1) use 5' trapping or 3' trapping; 2) use electroporation-based trapping or retrovirus-based trapping.

3.1.1 5' trapping versus 3' trapping

In Wentland's original work, she used the 3' trapping (polyA trap) strategy. The bioinformatic analysis of the trapped sequences generated in her experiment has shown that half of the 3' RACE sequences did not match any

known genes, and therefore termed “novel”. Some of these RACE sequences were shown to be transcribed in normal tissues, suggesting that these proviral integrations were located in transcribed genes. More detailed analysis has shown that the other sequences are not functional genes, consisting of processed pseudogenes and partially duplicated genomic sequences. Moreover, there are very few papers describing 3’ trapping strategies and the alleles generated by them. So it is difficult to assess the mutagenicity of 3’ trapping vectors. The transcripts initiated in the gene-trap vector can sometimes capture cryptic splice acceptors and PolyA signals downstream of the integration sites of the trapping vector. Also, if alternative terminal exons are trapped, only the transcripts that utilize the trapped exons will be disrupted. It is reasonable to predict that some of the trapped clones might not disrupt any functional genes and thus will not cause any phenotype. But on the other hand, 3’ trapping does not require the trapped genes to express in the undifferentiated ES cells which increases the possibility of the trapping of genes that express later in the developmental process.

On the other hand, most of the gene-trap mutagenesis experiments in mouse ES cells published so far use 5’ trapping (promoter trap) strategy. Considerable amount of data has been accumulated so that comparisons of the relative efficiency of different 5’ trapping constructs can be made based on statistical analysis of the trapped loci (Hansen, Floss et al. 2003). Also the mutagenicity of 5’ trapping strategy has been well documented and a significant portion of the genes trapped by promoter trap vectors were found to express in early embryogenesis (Gajovic, Chowdhury et al. 1998). In many cases, disruption of these genes will cause severe phenotypes *in vivo*. However, the nature of the promoter gene-trap design restricts 5’ trapping to those genes expressed in undifferentiated ES cells. Also, some genes that express at low levels are unlikely to be trapped.

So at the beginning stage of my project, vectors for both 5’ trapping and 3’ trapping have been constructed and functionally tested in ES cells. For reasons that will be discussed later, the 5’ trapping was chosen to develop the final strategy.

3.1.2 Electroporation versus retroviral vector

Trapping vectors can be introduced into the genome by either electroporation or retroviral infection. As I discussed before, both methods have their advantages and limitations.

Electroporation of a linearized gene-trap vector directly into mammalian cells is both simple and highly reproducible. There is almost no limitation on the size and structure of the vector, which allows flexibility for sophisticated vector design. But the structure of the mutant alleles created by this method is unpredictable. The integrations are always accompanied by DNA concatermerization. Multiple copies of the gene-trap vector in one locus can make the identification of the mutations difficult.

Gene trap mutagenesis using a retroviral vector results in a single copy of the retrovirus integrating into one genomic locus, which makes the cloning of the virus insertion site by PCR based methods reliable. Retroviruses have a tendency to integrate into 5' portion of a gene and thus are more likely to generate null alleles. However the packaging size of a retrovirus is limiting, and the packaging efficiency will drop significantly with an increase in the size of the exogenous DNA insert. Also, the virus preparation and transfection process are complicated and time-consuming compared to the electroporation method. Finally, it is not easy to predict which sequences can be efficiently packaged.

So at the beginning stage of my project, vectors for both electroporation-based trapping and retrovirus-based trapping have been constructed and functionally tested in ES cells. For the reasons that will be discussed later, the retrovirus-based trapping was selected.

3.2 Results

3.1.1 Construction of the inducible mitotic recombination cell line

3.2.1.1 Modification of the inducible mitotic recombination cassettes

The two original inducible mitotic recombination cassettes, pL330 (Multi *lox*-*Hprt* ∆5') and pL341 (Multi *lox*-*Hprt* ∆3'), contain *Neo* and *Puro* selection markers flanked by three *lox* site variants: *lox*5171, *lox*2272 and either *lox*66 (Multi *lox*-*Hprt* ∆3') or *lox*71 (Multi *lox*-*Hprt* ∆5') sites (Fig. 3-1) (Liu, Jenkins et al. 2002). A wild-type *lox*P site will be generated by site-specific recombination between *lox*66 and *lox*71 sites (Fig. 3-2a), which might recombine with the wild-type *lox*P sites used for regional trapping. Therefore *lox*66 or *lox*71 need to be deleted from the cassettes (Fig. 3-2b). Two pairs of oligonucleotides were used to generate the construct, pWW37 (Fig. 3-2c), which has *lox*5171, *lox*2272 and FRT sites flanked by several convenient enzyme cutting sites for the future cloning steps. The modified *lox* sites were ligated with *Neo*, *Puro*, 5' *Hprt* and 3' *Hprt* cassettes to make the modified inducible mitotic recombination cassettes, pWW48 (multi *lox* sites flanked *Puro*-3' *Hprt*) and pWW49 (5' *Hprt*-multi *lox* sites flanked *Neo*).

To fully sequence the two modified inducible mitotic recombination cassettes, pWW48 and pWW49 plasmids were digested with various restriction enzymes and subcloned into pBS. The sequencing results confirmed the intactness of the *lox* sites flanking the selection cassettes.

The *Neo*, *Puro*, 5' *Hprt* and 3' *Hprt* cassettes in pWW48 and pWW49 were functionally tested in ES cells. The linearized pWW48 and pWW49 plasmids were co-electroporated into AB2.2 ES cells lines with or without supercoiled pCAAG-Cre plasmid. The transformants were selected in M15 supplemented with HAT, G418 or puromycin, respectively. HAT resistant colonies were only generated when the two cassettes were co-electroporated with the Cre expression plasmid. Puromycin and G418 resistant colonies were generated by pWW48 and pWW49 as expected. The function test has proved the intactness of all the cassettes.

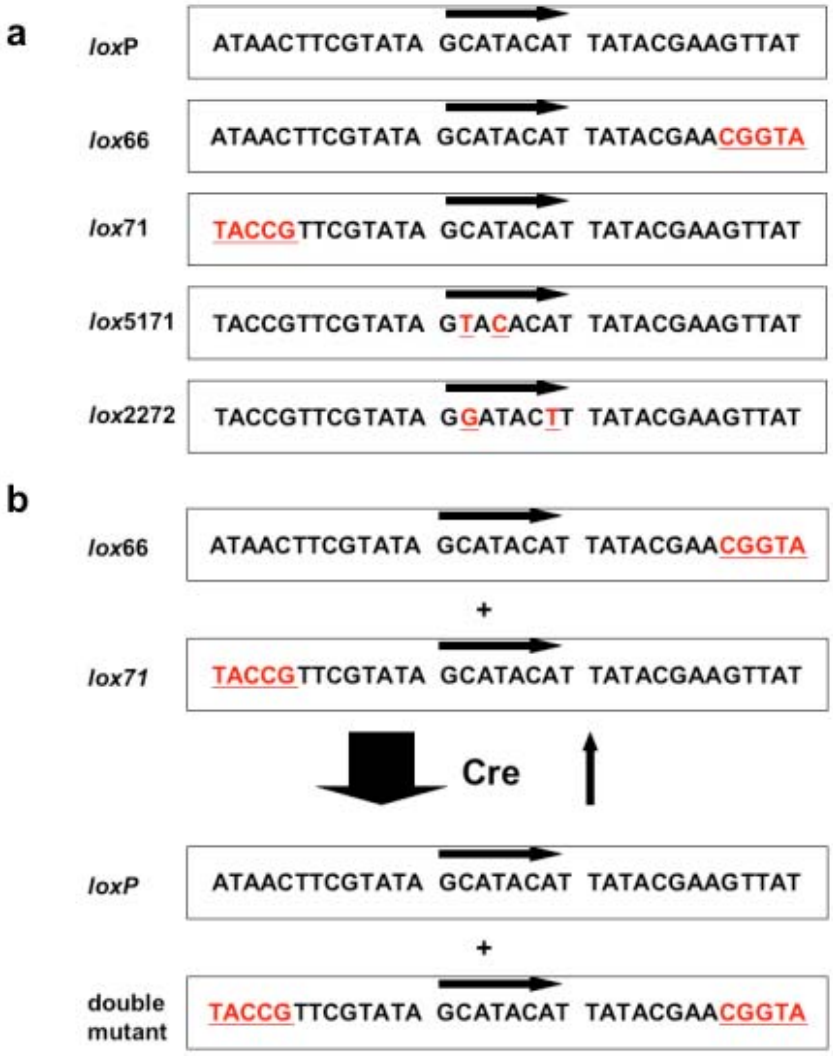


Fig. 3-1 *lox* sites used in the experiment. a. Nucleotide sequences of *lox* sites used in the experiment. The mutated nucleotides in the *lox* variants are underlined and marked as red. **b.** Site-spacific recombination between *lox*66 and *lox*71 sites. The Cre-mediated recombination between *lox*66 and *lox*71 will create a wild type *lox*P site, as well as a double mutant *lox* site.

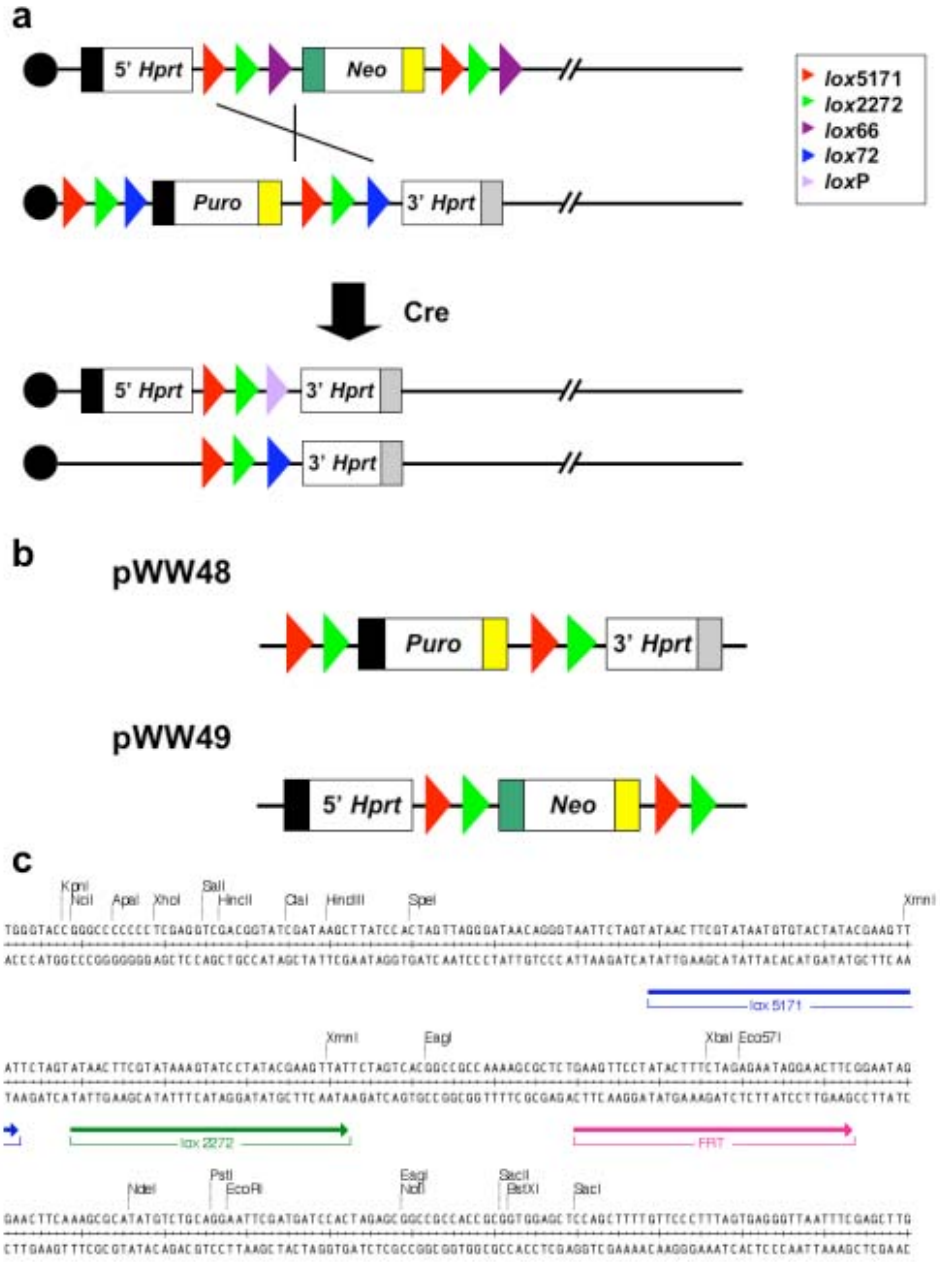

a
5' Hprt
Neo
Puro
3' Hprt
lox5171
lox2272
lox66
lox72
loxP
Cre
5' Hprt
3' Hprt
3' Hprt
b
pWW48
Puro
3' Hprt
pWW49
5' Hprt
Neo
c
KpnI
NcoI
ApaI
XhoI
SalI
HincII
ClaI
HindIII
SpeI
XmnI
TGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTATCCACTAGTTAGGGATAACAGGGTAATTCTAGTATAACTTCGTATAATGTGTACTATACGAAGTT
ACCCATGGCCCGGGGGGGAGCTCCAGCTGCCATAGCTATTCGAATAGGTGATCAATCCCTATTGTCCCATTAAGATCATATTGAAGCATATTACACATGATATGCTTCAA
lox5171
XmnI
EagI
XbaI
Eco57I
ATTCTAGTATAACTTCGTATAAAGTATCCTATACGAAGTTATTCTAGTCACGGCCGCCAAAAGCGCTCTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAG
TAAGATCATATTGAAGCATATTTCATAGGATATGCTTCAATAAGATCAGTGCCGGCGGTTTTCGCGAGACTTCAAGGATATGAAAGATCTCTTATCCTTGAAGCCTTATC
lox2272
FRT
NdeI
PstI
EcoRI
EagI
NotI
SacII
BstXI
SacI
GAACTTCAAAGCGCATATGTCTGCAGGAATTCGATGATCCACTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTTCGAGCTTG
CTTGAAGTTTCGCGTATACAGACGTCCTTAAGCTACTAGGTGATCTCGCCGGCGGTGGCGCCACCTCGAGGTCGAAAACAAGGGAAATCACTCCCAATTAAAGCTCGAAC

Fig. 3-2 Induced mitotic recombination cassettes. a. Induced mitotic recombination cassettes described in Liu et al. (2002). The multi *lox*-5' *Hprt* cassette contains a *PGK* promoter (black box), 5' half of the *Hprt* minigene, tandem *lox* site variants, a *Pol*II promoter(green box) that drives *Neo* expression, and a bovine growth hormone polyA site (yellow box). The multi *lox*-3' *Hprt* cassette contains a *PGK* promoter (black box) that drives *Puro* expression, a bovine growth hormone polyA site (yellow box), tandem *lox* site variants, 3' half of the *Hprt* minigene and an SV40 polyA site (gray box). Arrows of different colour denote variant *lox* sites. Note that Cre-induced mitotic recombination reconstitutes a wild type *Hprt* minigene and leaves a wild type *lox*P site (purple arrow) on the same chromosome as the 3' *Hprt* cassette. **b.** Modified induced mitotic recombination cassettes with multi *lox* sites. The structure of the modified cassettes is essentially the same as the original cassettes, except that the *lox*66 and *lox*71 sites were deleted. These mutant *lox* sites will not recombine with each other or with wild type *lox*P site. **c.** Sequence of the tandem *lox* sites. Two pairs of oligos were used to construct the tandem *lox* sites. The *lox* sites were used to flank the *Puro* and *Neo* selection markers so that the selection markers can be popped-out afterwards. The tandem *lox* sites can also increase the efficiency of the induced mitotic recombination.

3.2.1.2 Targeting of the inducible mitotic recombination cassettes

After all the selection markers and the *lox* sites in pWW48 and pWW49 were confirmed to be functional *in vivo*, the 5' *Hprt* and 3' *Hprt* cassettes were cloned into pL325 to make the final targeting vectors, pWW74 (3' *Hprt*) and pWW75 (5' *Hprt*). The two vectors were linearized and co-electroporated into AB2.2 ES cells with or without pCAAG-Cre plasmid to confirm the functionality of the selection cassettes. HAT resistant colonies were only recovered from the co-transformation with the Cre expression plasmid, which proved that the two cassettes were functional (data not shown).

Linearized pWW74 was electroporated into AB2.2 ES cells and the transformants were selected with puromycin and FIAU. The correctly targeted clones were identified by Southern analysis using a *D11Mit71* 3' probe. The expected sizes of the detected restriction fragments were 14.1 kb for the wild-type allele and 10.3 kb for the targeted allele. The correctly targeted clones were expanded and confirmed by Southern analysis using a *D11Mit71* 5' probe. The expected sizes of detected restriction fragments were 7.1 kb for the wild-type allele and 4.9 kb for the targeted allele when digested with *Xba*I (Fig. 3-3). The targeted clones were named as WW14.

Linearized pWW75 was electroporated into AB2.2 ES cells and the transformants were selected with G418 and FIAU. The correctly targeted clones were identified by Southern analysis using a *D11Mit71* 3' probe. The expected sizes of detected restriction fragments were 14.1 kb for the wild-type allele and 17.9 kb for the targeted allele. The correctly targeted clones were expanded and confirmed by Southern analysis using a *D11Mit71* 5' probe. The expected sizes of detected restriction fragments were 7.1 kb for the wild-type allele and 6.4 kb for the targeted allele when digested with *Xba*I (Fig. 3-4). The targeted clones were named as WW16.

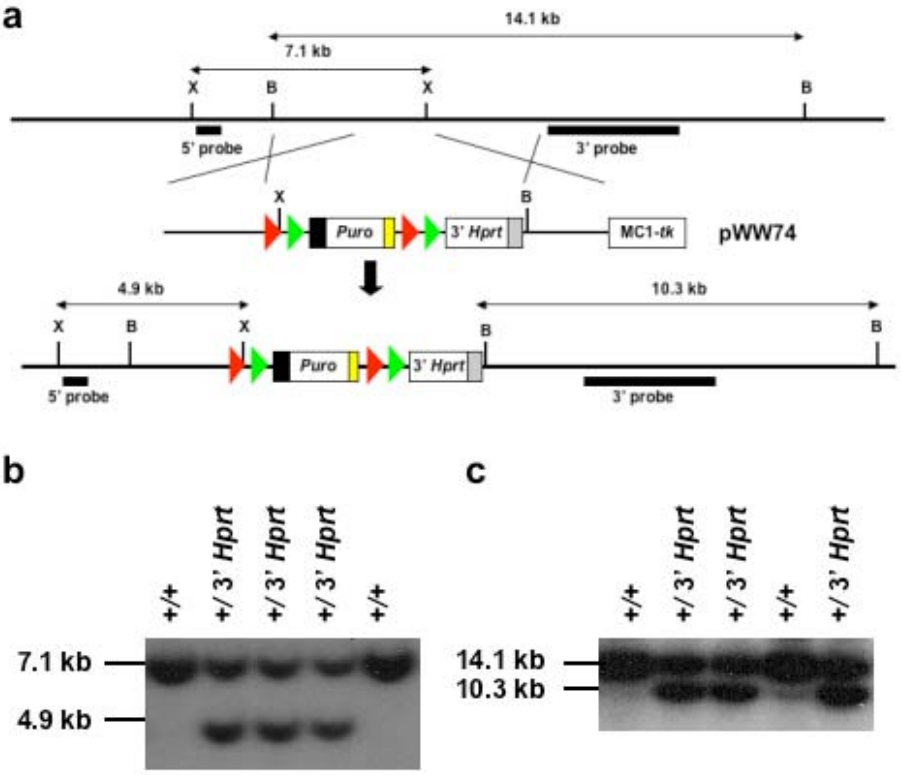


Fig. 3-3 Targeting 3' *Hprt* cassette to *D11Mit71* locus. a. Schematic illustration of targeting 3' *Hprt* cassette to *D11Mit71* locus. A 2.1 kb genomic fragment at *D11Mit71* locus was replaced by the 3' *Hprt* cassette (pWW48) to make the 3' *Hprt* targeting vector (pWW74). The MCI-*tk* cassette was used as the negative selection marker. Arrows of different colours denote multi *lox* sites used to flank the *Puro* selection cassettes. B, *Bam*HI; X, *Xba*I. **b.** Southern analysis of the WW14 cell line using a *D11Mit71* 5' probe. Genomic DNA was digested with *Xba*I and hybridized with a *D11Mit71* 5' probe, the detected restriction fragments were 7.1 kb for the wild type allele and 4.9 kb for the targeted allele. **c.** Southern analysis of the WW14 cell line using a *D11Mit71* 3' probe. Genomic DNA was digested with *Bam*HI and hybridized with a *D11Mit71* 3' probe, the detected restriction fragments were 14.1 kb for the wild type allele and 10.3 kb for the targeted allele.

To confirm the pluripotency of the cell lines, several subclones from WW14 and WW16 cell lines were injected into C57^{TyrBrdC1} blastocysts and germline transmissions were obtained for both of them. Germline transmission of the targeted alleles was confirmed by Southern analysis using the restriction enzymes and probes described above. Mice carrying the targeted alleles were crossed to a Cre expression mouse line (Su, Mills et al. 2002) and a FLP-expressing mouse line (Farley, Soriano et al. 2000; Su, Mills et al. 2002). The multi *lox* sites flanked *Neo* and *Puro* cassettes can be popped out in mice which carry both the inducible mitotic recombination cassettes and the Cre or Flp transgene. The two mouse lines can be crossed together to generate genetic mosaics *in vivo* by induced mitotic recombination (data not shown).

To make a cell line that carries both the 5' *Hprt* and the 3' *Hprt* cassettes targeted to allelic positions at the *D11Mit71* locus on chromosome 11, linearized pWW75 was electroporated into WW14. The transfectants were selected with G418 and FIAU and the correctly targeted clones were identified by Southern analysis using a *D11Mit71* 3' probe. The double-targeted clones were expected to have 10.3 kb (3' *Hprt* targeting) and 17.9 kb (5' *Hprt* targeting) *Bam*HI restriction fragments. The correctly targeted clones were expanded and confirmed by Southern analysis using a *D11Mit71* 5' probe. The double-targeted clones were expected to have 4.9 kb band (3' *Hprt* targeting) and 6.4 kb band (5' *Hprt* targeting) *Xba*I restriction fragments. The double-targeted ES cell clones were named as WW24 (Fig. 3-5a & b). Similarly, linearized pWW74 was electroporated into WW16 to make the double-targeted cell line WW25. The Southern screening strategy and the sizes of the detected restriction fragments are the same as described for WW24.

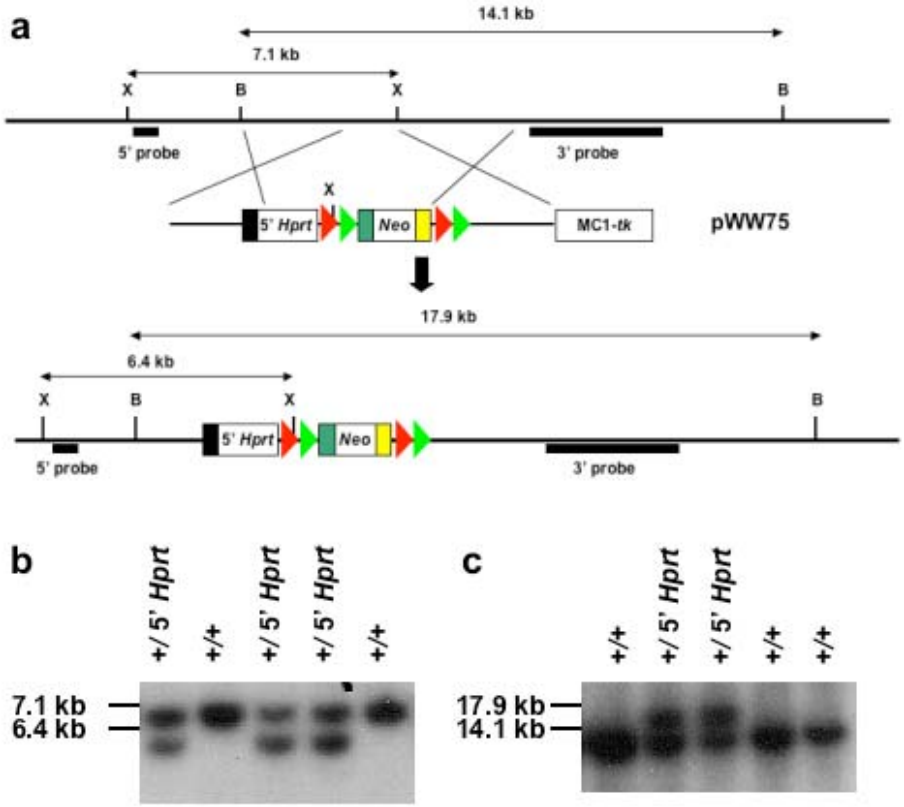


Fig. 3-4 Targeting 5' *Hprt* cassette to *D11Mit71* locus. a. Schematic illustration of targeting 5' *Hprt* cassette to *D11Mit71* locus. A 2.1 kb genomic fragment at *D11Mit71* locus was replaced by the 5' *Hprt* cassette (pWW49) to make the 5' *Hprt* targeting vector (pWW75). The MCI-*tk* cassette was used as the negative selection marker. Arrows of different colours denote multi *lox* sites used to flank the *Neo* selection cassettes. B, *Bam*HI; X, *Xba*I. **b.** Southern analysis of the WW16 cell line using a *D11Mit71* 5' probe. Genomic DNA was digested with *Xba*I and hybridized with a *D11Mit71* 5' probe, the detected restriction fragments were 7.1 kb for the wild type allele and 6.4 kb for the targeted allele. **c.** Southern analysis of the WW16 cell line using a *D11Mit71* 3' probe. Genomic DNA was also digested with *Bam*HI and hybridized with a *D11Mit71* 3' probe, the detected restriction fragments were 14.1 kb for the wild type allele and 17.9 kb for the targeted allele.

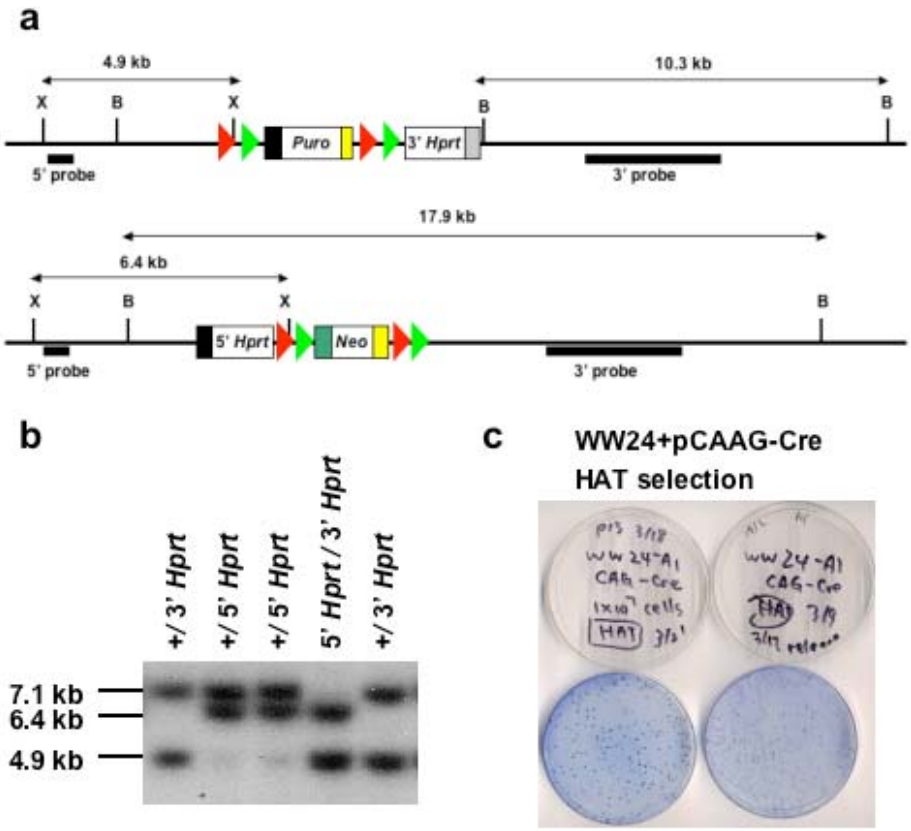


Fig. 3-5 Generating the *D11Mit71*$^{5' Hprt/3' Hprt}$ ES cell line. a. Schematic illustration of double-targeted *D11Mit71* alleles. Arrows of different colours denote multi *lox* sites used to flank the *Neo* and *Puro* selection cassettes. B, *Bam*HI; X, *Xba*I. **b.** Southern analysis of the WW24 cell line. Genomic DNA was digested with *Xba*I and hybridized with a *D11Mit71* 5' probe, the detected restriction fragments for double targeted clones were 6.4 kb for the 5' *Hprt* targeted allele and 4.9 kb for the 3' *Hprt* targeted allele. For some clones, the 5' *Hprt* cassette targeting resulted into the replacement of the 3' *Hprt* targeted allele, instead of the wild type allele. **c.** Functional test of the inducible mitotic recombination cassettes. pCAAG-Cre plasmid was electroporated into WW24 ES cell and the recombinants were selected by M15 supplemented with HAT. HAT resistant colonies were recovered as expected. The experiment was repeated twice.

To test the function of the 5' *Hprt*, 3' *Hprt* and the *lox* sites, pCAAG-Cre plasmid was electroporated into WW24 and WW25 cells. HAT resistant colonies were recovered from both cell lines when the Cre was transiently expressed. This experiment confirmed the functional intactness of the 5' *Hprt*, 3' *Hprt* and the *lox* sites (Fig. 3-5c).

To pop-out the *lox* site flanked *Neo* and *Puro* cassettes from WW24, pCAAG-Cre was electroporated into WW24 cells (Fig. 3-6). The cells were plated at low density and allowed to form single colonies without drug selection. The clones that lost both *Neo* and *Puro* selection markers were identified by sib-selection using M15, M15+G418, M15+puromycin and M15+HAT, respectively. The correct recombinants should be G418 sensitive, puromycin sensitive and HAT sensitive (Fig. 3-6). The function of the 5' *Hprt* and 3' *Hprt* cassettes were tested by transient Cre expression and subsequent HAT selection (Fig. 3-7a). The appropriate clones were expanded and confirmed by Southern analysis using a *D11Mit71* 5' probe. The popped-out double-targeted clones were expected to have 4.9 kb band (3' *Hprt* targeting) and 6.4 kb band (5' *Hprt* targeting) *Xba*I restriction fragments (Fig. 3-7b).

3.2.1.3 Targeting of the end point cassette for regional trapping

The original regional trapping design of Wentland et al. (unpublished data) utilized the two non-functional half *Hprt* cassettes to select for inversion events (Fig. 3-8a). Since we have already used these cassettes for inducible mitotic recombination, we elected to use the split promoter and selection marker strategy to achieve efficient recovery of inversion events. In brief, a *PGK-lox*P-*Bsd* cassette was first targeted to the E_2DH locus to fix the inversion end point. A promoter-less, non-functional *lox*P-*Puro* cassette was then introduced by retroviral integration. By Cre-mediated site-specific recombination, the two cassettes recombined to produce a functional *PGK-lox*P-*Puro* cassette which will be resistant to puromycin selection (Fig. 3-8b).

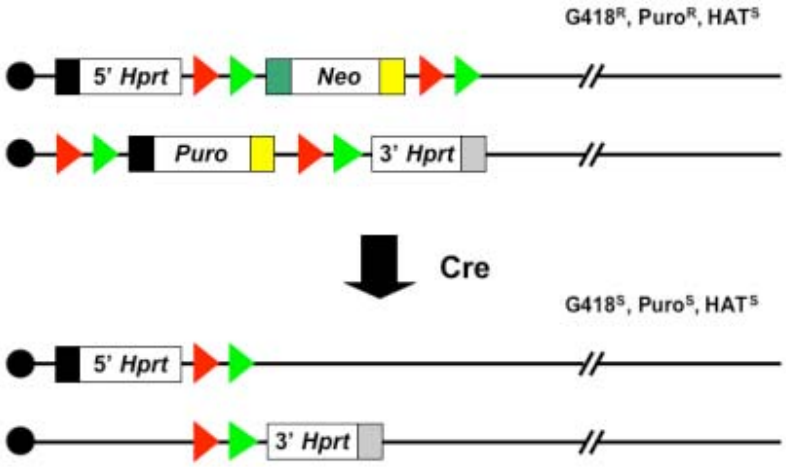


Fig. 3-6 Selection marker pop-out of the *D11Mit71*$^{5'\ Hprt/3'\ Hprt}$ ES cell line. Schematic illustration of selection marker pop-out of the double-targeted *D11Mit71* alleles. Arrows of different colour denote multi *lox* sites used to flank the *Neo* and *Puro* selection cassettes.

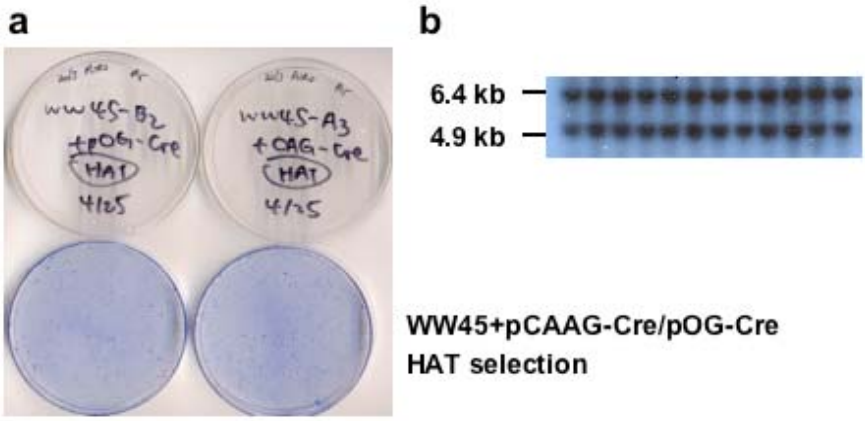


Fig. 3-7. **Test of the WW45 cell line a.** Functional test of the induced mitotic recombination cassettes. pCAAG-Cre or pOG-Cre plasmid was electroporated into WW45 ES cells and the recombinants were selected by M15 supplemented with HAT. HAT resistant colonies were recovered as expected. The experiment was repeated twice. **b.** Southern analysis of the WW45 cell line. Genomic DNA was digested with *Xba*I and hybridized with a *D11Mit71* 5' probe, all the Puro^s G418^s clones carry the 6.4 kb restriction fragment for the 5' *Hprt* targeted allele and 4.9 kb restriction fragment for the 3' *Hprt* targeted allele.

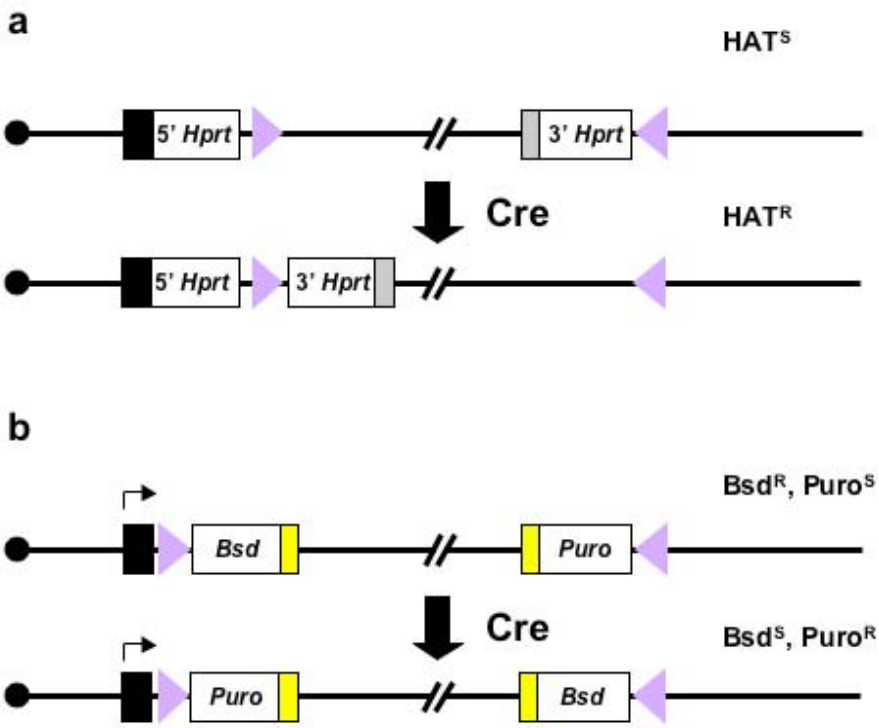
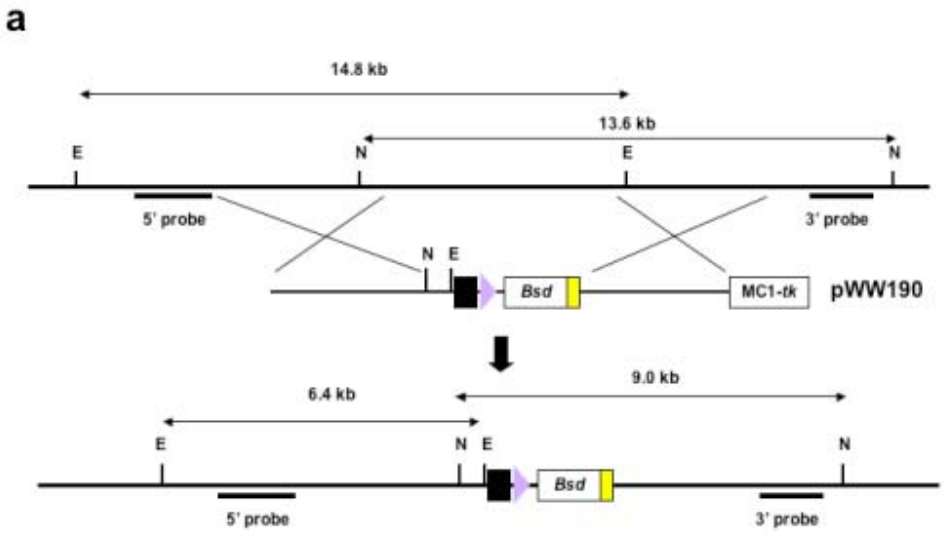


Fig. 3-8 Regional trapping strategy. a. Schematic illustration of the original regional trapping selection strategy by Wentland *et al.* (unpublished data). The Cre-mediated recombination can reconstitute a functional *Hprt* gene. **b.** Schematic illustration of the regional trapping selection strategy used in my project. The Cre-mediated recombination will reconstitute a functional *Puro* cassette, and disrupt the original *Bsd* cassette. Black box, *PGK* promoter; yellow box, bovine growth hormone polyA site; gray box, SV40 polyA site; purple arrow, wild type *lox*P site.

A *PGK-lox*P-*EM7-Bsd-bpA* (pWW146) cassette was constructed by ligating a pair of oligonucleotides containing a wild-type *lox*P site into pL313 (*PGK-EM7-Bsd-bpA*). The function of this cassette was tested by electroporating the linearized plasmid DNA into AB2.2 ES cells and selected in M15 medium supplemented with blasticidin. Blasticidin resistant colonies were recovered from the selection, which proved that the insertion of the *lox*P site did not interfere with the function of the *Bsd* selection cassette. The intactness of the inserted *lox*P site between the *PGK* promoter and the *Bsd* ORF was confirmed by sequencing (data not shown).

After the cassette was tested *in vivo*, it was ligated with two E_2DH homology arms and a MC1-HSV*tk* negative selection marker to make the final targeting vector, pWW190. Linearized pWW190 was electroporated into WW45 cells and the transfectants were selected with blasticidin and FIAU. Correctly targeted clones were identified by Southern analysis (Fig. 3-9a). Genomic DNA was digested with *Eco*RI and hybridized with an E_2DH 5' probe. The targeted restriction fragment was 9.2 kb and the wild-type restriction fragment was 14.9 kb (Fig. 3-9b). Genomic DNA was also digested with *Nde*I and hybridized with an E_2DH 3' probe. The targeted restriction fragment was 9.6 kb and the wild-type restriction fragment was 13.1 kb (Fig. 3-9c). To confirm that the targeted clones also carried the two induced mitotic recombination cassettes, genomic DNA was digested with *Xba*I and hybridized with a *D11Mit71* 5' probe, all the blasticidin resistant clones had both a 6.4 kb restriction fragment (5' *Hprt* targeting and *Neo* pop-out) and a 5.0 kb restriction fragment (3' *Hprt* targeting and *Puro* pop out) (Fig. 3-9d). Two correctly targeted clones, WW69-C8 and WW69-D6, were identified by Southern analysis. Sib-selection was carried out to determine the functional intactness of all the cassettes using M15, M15+G418, M15+puromycin, M15+HAT and M15+blasticidin, respectively. The two clones were G418 sensitive, puromycin sensitive, HAT sensitive and blasticidin resistant (Fig. 3-9e).


a
14.8 kb
13.6 kb
E
N
E
N
5' probe
3' probe
N E
Bsd
MC1-tk
pWW190
6.4 kb
9.0 kb
E
N E
N
Bsd
5' probe
3' probe

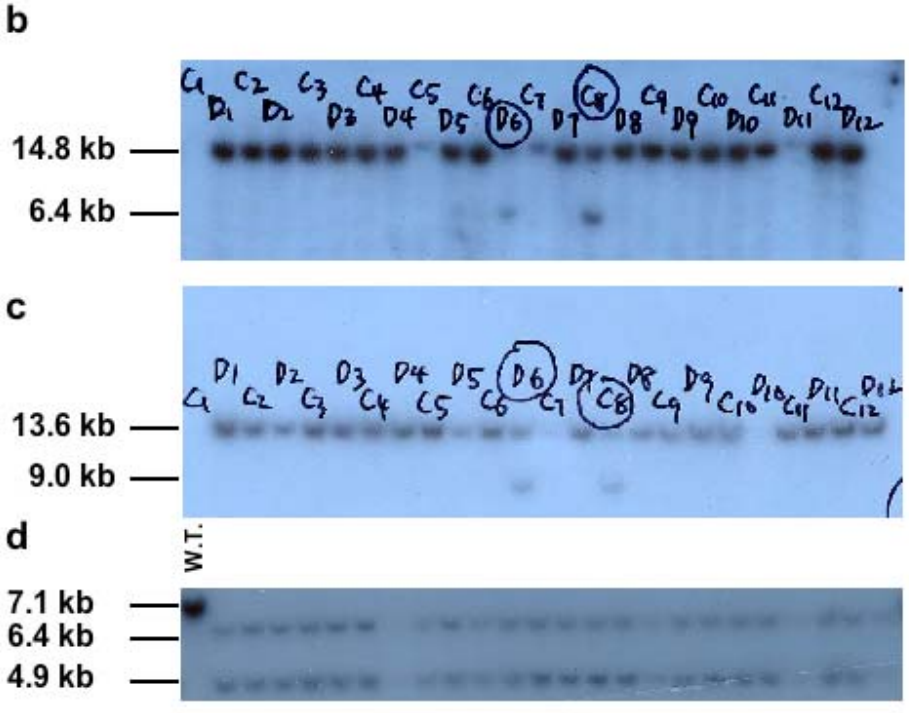

b
14.8 kb
6.4 kb
c
13.6 kb
9.0 kb
d
W.T.
7.1 kb
6.4 kb
4.9 kb

Fig. 3-9 Generating end-point targeting ES cell line. a. Schematic illustration of targeting the *PGK-lox*P-*Bsd-bpA* cassette to the E_2DH locus. Arrow denotes the wild-type *lox*P site used to separate the *PGK* promoter and the *Bsd* ORF. E, *Eco*RI; N, *Nde*I. **b.** Southern analysis of the WW69 cell line using a E_2DH 5' probe. Genomic DNA was digested with *Eco*RI and hybridized with an E_2DH 5' probe, the targeted allele was 6.4 kb and the wild type allele was 14.8 kb. **c.** Southern analysis of the WW69 cell line using a E_2DH 3' probe. Genomic DNA was digested with *Nde*I and hybridized with a E_2DH 3' probe, the targeted allele was 9.0 kb and the wild type allele was 13.6 kb. **d.** Southern analysis of the WW69 cell line using a *D11Mit71* 5' probe. Genomic DNA was digested with *Xba*I and hybridized with a *D11Mit71* 5' probe, the detected restriction fragments were 6.4 kb for the 5' *Hprt* targeted allele, 4.9 kb for the 3' *Hprt* targeted allele and 7.1 kb for the wild type allele. DNA from a wild type cell line was loaded on the left as a control.

To use induced mitotic recombination to make homozygous mutations, the disruption of the first allele should happen on the same chromosome as the 3' *Hprt* cassette. A direct way to determine the location of the end point targeting cassette is to induce mitotic recombination by transient Cre expression, and check the genotypes of the recombinants recovered. If most of the HAT resistant colonies are homozygous for the targeted *E_2DH* allele, the end point targeting cassette is targeted to the right chromosome (Fig. 3-10a). On the other hand, if no clone is homozygous for targeted *E_2DH* allele, the end point targeting cassette is targeted to the wrong chromosome (Fig. 3-10b).

To determine the location of the *PGK-lox*P-*Bsd-bpA* cassette, a Cre expression plasmid was electroporated into WW69-C8 and D6 cell lines and transfectants were selected in M15 supplemented with HAT. 36 HAT resistant colonies were picked from each electroporation and Southern analysis was performed using the *D11Mit71* 5' probe, *D11Mit71* 3' probe, *E_2DH* 5' probe and *E_2DH* 3' probe, respectively (Fig. 3-11). Sib-selection was carried out to determine the drug resistance of each recombinant using M15, M15+HAT and M15+blasticidin, respectively. Clones that carried two targeted *E_2DH* alleles were recovered from recombinants of WW69-D6. Of the 25 clones that carried at least one copy of *PGK-lox*P-*Bsd-bpA* cassette (resistant to blasticidin selection), 18 of them were homozygous for targeted *E_2DH* locus (X segregation) and 7 of them were heterozygous (Z segregation). The percentage of G2-X segregation is 72%, which is comparable to the data described before (Liu, Jenkins et al. 2002). 2 of the clones that were homozygous for the targeted *E_2DH* allele also carried two copies of 5' *Hprt* cassettes, suggesting that the induced mitotic recombination was followed by second around of mitotic recombination between the centromere and the *D11Mit71* locus or a chromosome loss/duplication event (Fig. 3-12).

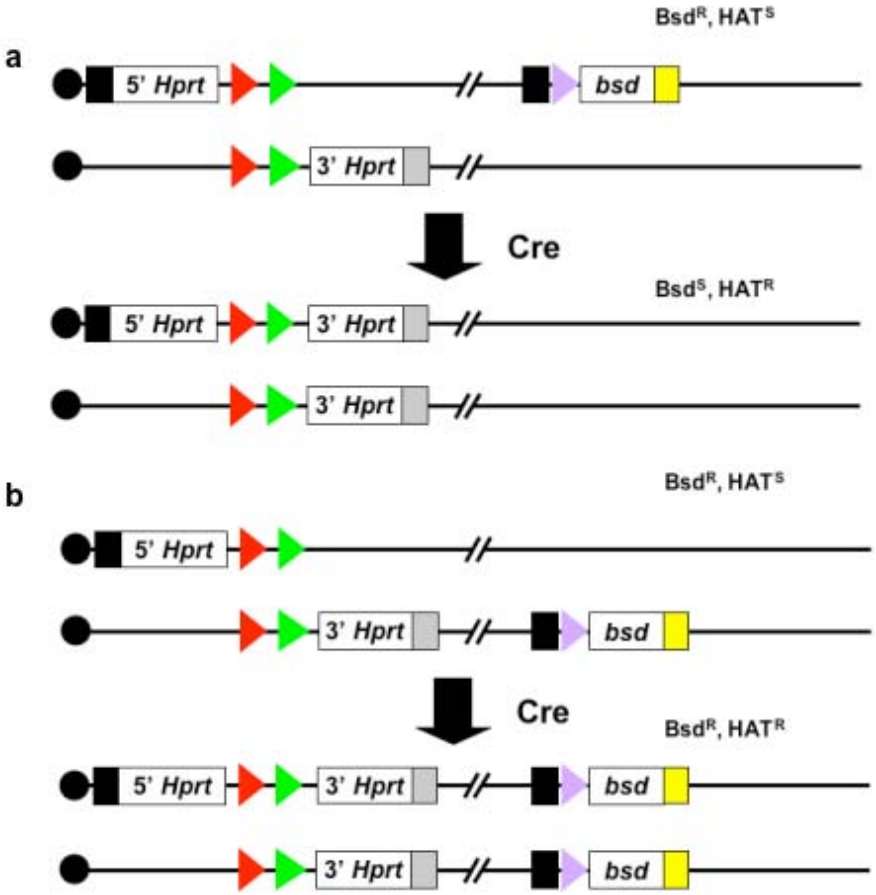


Fig. 3-10 The location of the end point targeting cassette. a. Schematic illustration of the outcome of the induced mitotic recombination when the end-point targeting cassette is on the same chromosome as the 5' *Hprt* cassette. Note that only G2-X segregation is shown here, no clones homozygous for the targeted E_2DH allele will be recovered in this situation. **b.** Schematic illustration of the outcome of the induced mitotic recombination when the end-point targeting cassette is on the same chromosome as the 3' *Hprt* cassette. Note that only G2-X segregation is shown here, clones homozygous for the targeted E_2DH allele will be recovered in this situation. Black box, *PGK* promoter; yellow box, bovine growth hormone polyA site; gray box, SV40 polyA site; arrows of different colors denote wild type and mutant *lox*P sites.

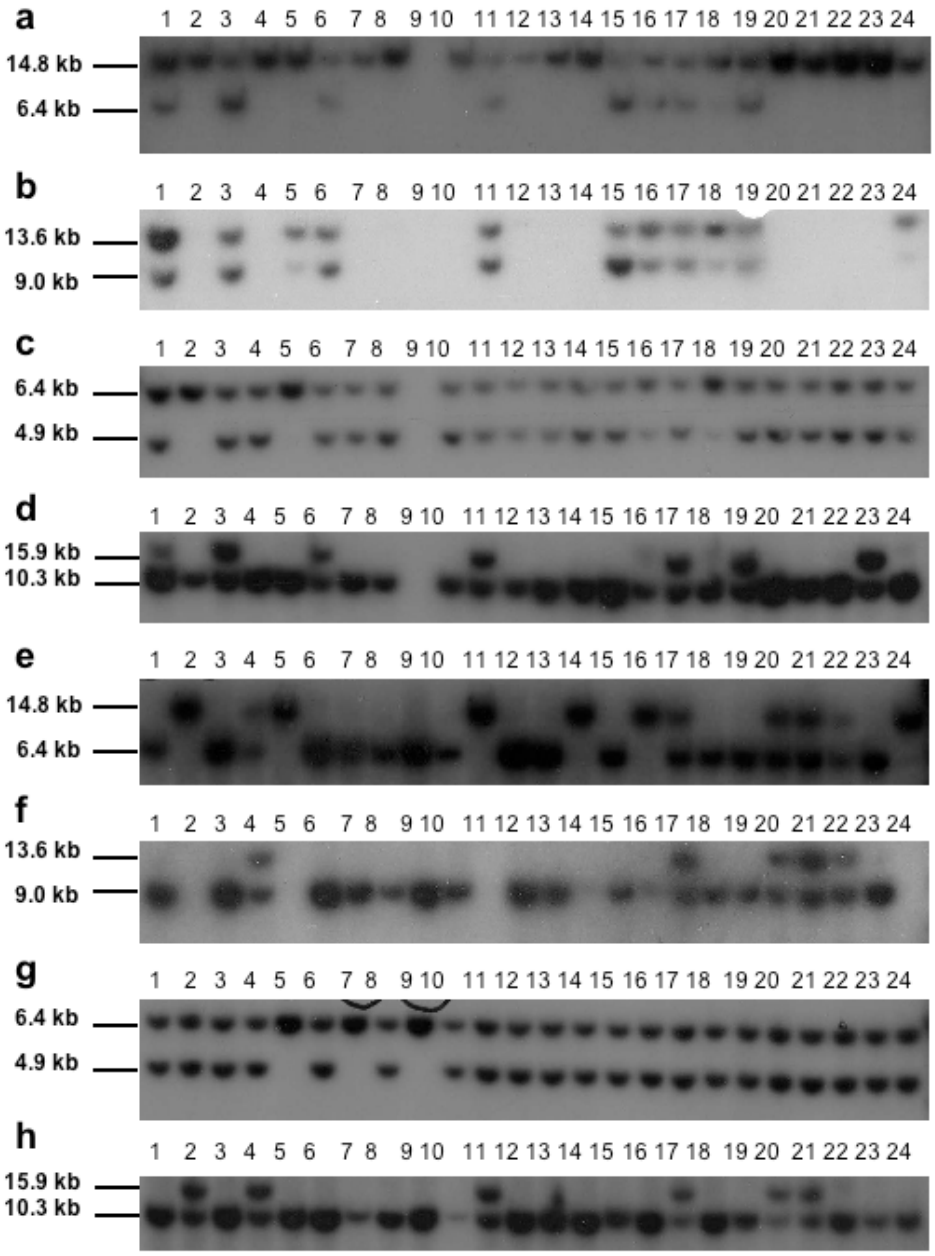

a
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
14.8 kb
6.4 kb
b
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
13.6 kb
9.0 kb
c
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
6.4 kb
4.9 kb
d
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
15.9 kb
10.3 kb
e
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
14.8 kb
6.4 kb
f
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
13.6 kb
9.0 kb
g
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
6.4 kb
4.9 kb
h
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
15.9 kb
10.3 kb

Fig. 3-11 Determination of the location of the end point targeting cassette by induced mitotic recombination. a. Genomic DNA from WW69-C8 recombinants was digested by *Eco*RI and hybridized with a E_2DH 5' probe, the targeted restriction fragment was 9.2 kb and the wild type restriction fragment was 14.9 kb. **b.** Genomic DNA from WW69-C8 recombinants was digested by *Eco*RI and hybridized with a E_2DH 3' probe, the targeted restriction fragment was 9.6 kb and the wild type restriction fragment was 13.1 kb. **c.** Genomic DNA from WW69-C8 recombinants was digested by *Xba*I, hybridized with a *D11Mit71* 5' probe, the 5' *Hprt* cassette targeted fragment was 6.4 kb and 3' *Hprt* cassette targeted fragment was 4.9 kb fragment . **d.** Genomic DNA from WW69-C8 recombinants was digested by *Bam*HI, hybridized with a *D11Mit71* 3' probe, the 5' *Hprt* cassette targeted fragment was 10.3 kb and 3' *Hprt* cassette targeted fragment was 15.9 kb. **e.** Genomic DNA from WW69-D6 recombinants was digested by *Eco*RI and hybridized with a E_2DH 5' probe, the targeted restriction fragment was 9.2 kb and the wild type restriction fragment was 14.9 kb. **f.** Genomic DNA from WW69-D6 recombinants was digested by *Eco*RI and hybridized with a E_2DH 3' probe, the targeted restriction fragment was 9.6 kb and the wild type restriction fragment was 13.1 kb. **g.** Genomic DNA from WW69-D6 recombinants was digested by *Xba*I, hybridized with a *D11Mit71* 5' probe, the 5' *Hprt* cassette targeted fragment was 6.4 kb and 3' *Hprt* cassette targeted fragment was 4.9 kb fragment . **h.** Genomic DNA from WW69-D6 recombinants was digested by *Bam*HI, hybridized with a *D11Mit71* 3' probe, the 5' *Hprt* cassette targeted fragment was 10.3 kb and 3' *Hprt* cassette targeted fragment was 15.9 kb. Note that for **b** and **f**, the Southern analysis was carried out on blasticidine resistant clones. So all the clones that are sensitive to the drug were killed during the selection.

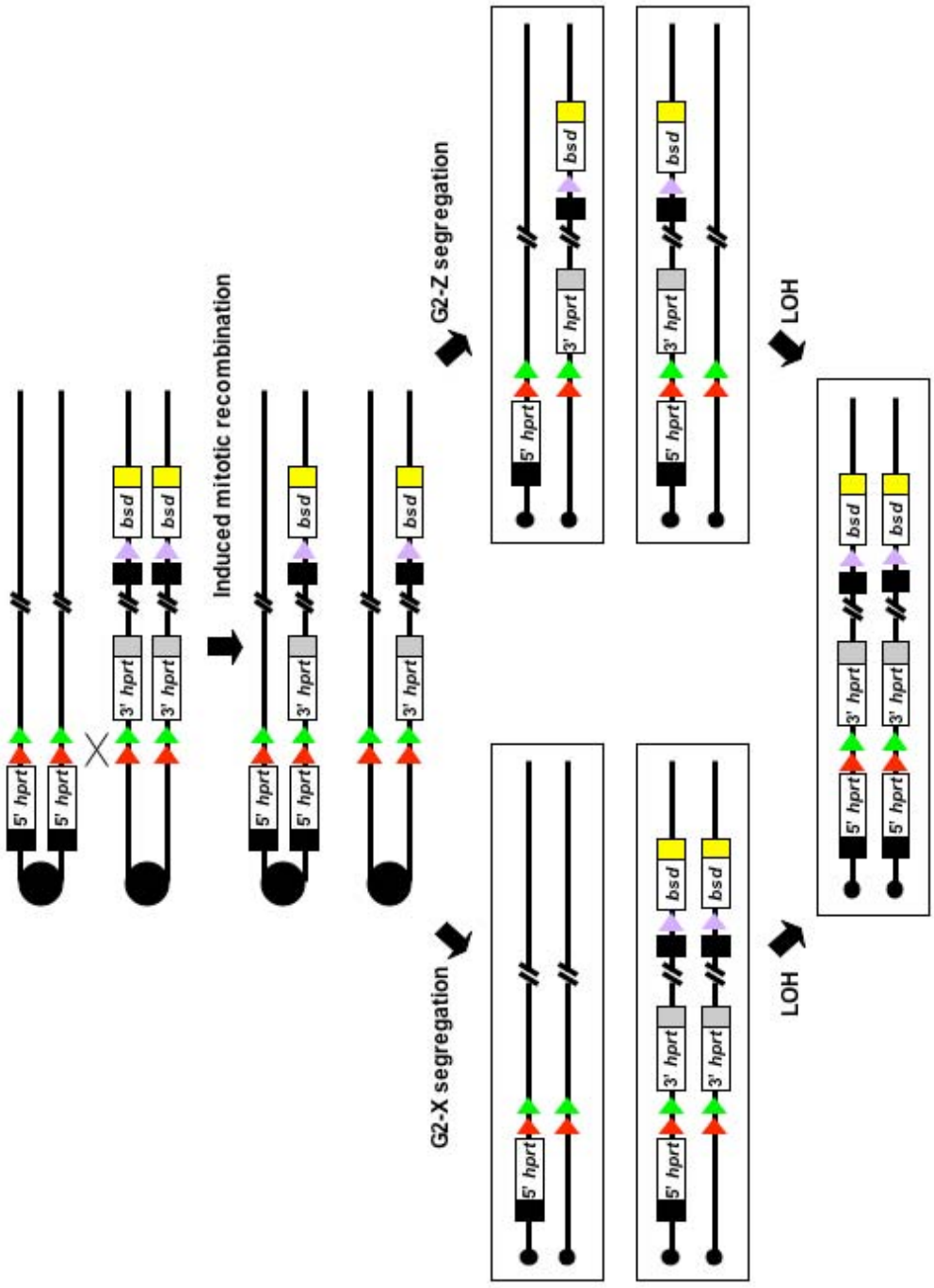

5' hprt
3' hprt
bsd
Induced mitotic recombination
G2-Z segregation
G2-X segregation
LOH
LOH

Fig. 3-12 Segregation pattern of recombinant chromatides after G2 recombination. Note that G2-X segregation will make the *PKG-lox*P-*bsd-bpA* cassette homozygous. But some HAT resistant clones carry two 5' *hprt* cassettes, two 3' *hprt* cassettes and two *PKG-lox*P-*bsd-bpA* cassettes. These cones might derive from the LOH event or a second mitotic recombination following the induced mitotic recombination. Black box, *PGK* promoter; yellow box, bovine growth hormone polyA site; gray box, SV40 polyA site; arrows of different colors denote wild type and mutant *lox*P sites.

11 clones were sensitive to blasticidin and carried two wild-type *E_2DH* alleles, suggesting that they derive either from untargeted WW45 cells carried over with *PGK-lox*P-*Bsd-bpA* targeted WW69 cells or a spontaneous mitotic recombination occurred somewhere between the *D11Mit71* and *E_2DH* loci before the Cre-induced mitotic recombination. Since the spontaneous mitotic recombination rate is very low (10^{-7} to 10^{-8}), these clones are most likely derived from untargeted WW45 cell contamination. To eliminate this background, WW69-D6 cells were plated at low density to form single colonies. The subclones were confirmed by Southern analysis and used for future experiments.

For WW69-C8, no HAT resistant colonies were homozygous for the targeted *E_2DH* allele, therefore the *PGK-lox*P-*Bsd-bpA* cassette was targeted to the wrong chromosome. Linearized pWW190 was also electroporated into another *D11Mit71*$^{5'\ Hprt/3'\ Hprt}$ cell line, WW46. Several targeted clones were identified using the same Southern screening strategy. But the subsequent Cre-induced mitotic recombination proved that for all these clones, the *PGK-lox*P-*Bsd-bpA* cassette was also targeted to the wrong chromosome (data not shown). Therefore, all the future experiments were carried out in WW69-D6 cell line.

3.2.2 Construction of the regional trapping vectors

The original gene-trap retrovirus of Wentland et al. (unpublished data) contained a 3' trapping cassette and a *lox*P-3' *Hprt* cassette for the selection of inversions. Since we elected to use the split promoter and selection marker strategy to select for the regional inversion events, some modifications were required to the original virus. A 5' trapping retrovirus was also constructed to compare the trapping efficiency and the subsequent inversion efficiency.

3.2.2.1 5' trapping vectors

The 5' trapping virus (pWW239) has a SAβ*geo* cassette (Friedrich and Soriano 1991) and a promoter-less *lox*P-*Puro-bpA* cassette (Fig. 3-13a). To test the function of the two cassettes, linearized pWW239 and pWW183 (*PGK-lox*P-*EM7-Bsd-bpA*) plasmids were co-electroporated into wild-type

AB2.2 ES cells with or without a Cre expression plasmid, and the transfectants were selected with M15+G418, M15+blasticidin, or M15+puromycin, respectively.

As a control, linearized pWW239 plasmid was also electroporated into AB2.2 and selected with M15+G418, or M15+puromycin, respectively. G418 resistant colonies were recovered, which confirmed that the 5' trapping cassette worked in ES cells. No puromycin resistant colonies were recovered when pWW239 was electroporated into AB2.2 ES cells alone. This result confirmed that promoter-less *lox*P-*Puro*-*bpA* cassette of the virus does not function. Importantly, puromycin resistant colonies were only recovered when linearized pWW239 and pWW183 (*PGK*-*lox*P-*EM7*-*Bsd*-*bpA*) plasmids were co-electroporated into AB2.2 ES cells with the Cre expression plasmid. This result confirms that when the non-functional *Puro* cassette gains the *PGK* promoter by Cre-mediated recombination, it becomes functional. This test has confirmed that both halves of the virus were functional *in vivo* (Fig. 3-14). This virus construct was used for future experiments.

The size of the exogenous DNA fragment that can be inserted into the virus backbone is limited, and the virus packaging efficiency drops significantly when the size of the virus increases. The two cassettes in the pMSCV (Clontech) virus backbone are about 5.3 kb in size, so it is reasonable to predict that the trapping titre will be low. Compared with the virus-based trapping vectors, there is little limit on the size of the electroporation-based trapping vectors. So electroporation-based 5' trapping vectors (pWW237) were also constructed. The two cassettes in pWW237 are essentially the same as in pWW239. However, instead of cloning these two cassettes into a virus backbone, they were cloned into pBS vector. pWW237 was functionally tested in the same way as pWW239. The results confirmed that the trapping and inversion cassettes were functional *in vivo*.

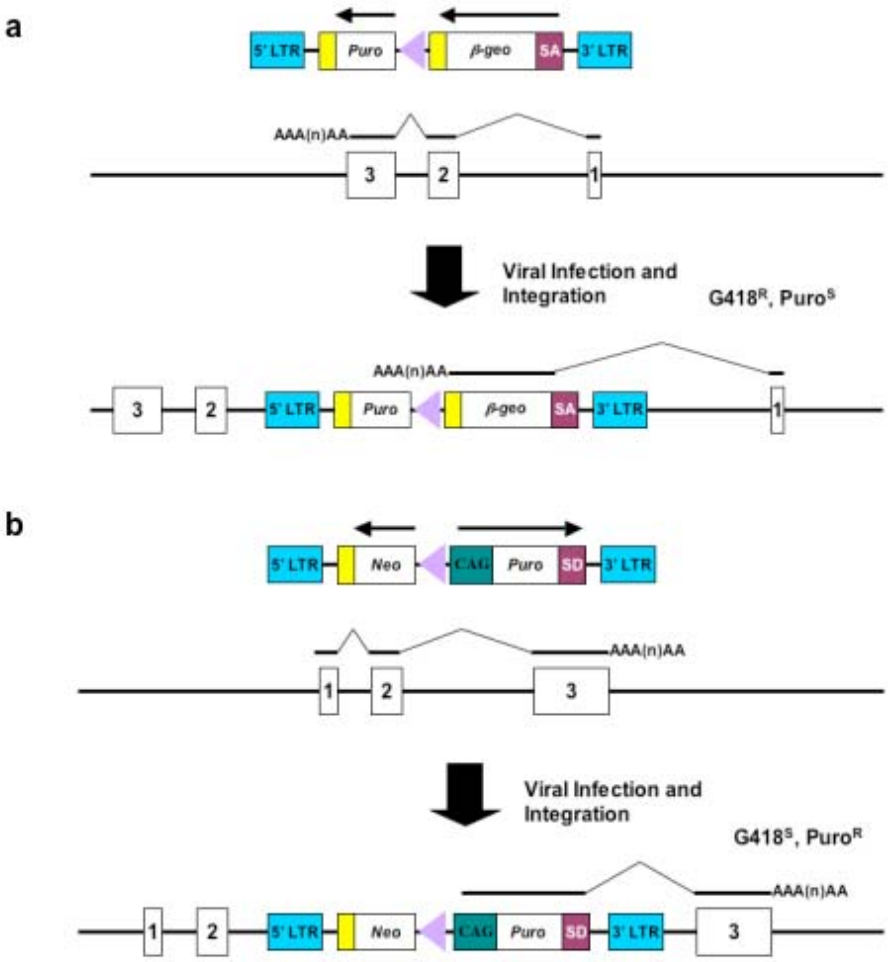


Fig. 3-13 The structure of gene trapping virus. a. Schematic illustration of the structure of the 5' trapping virus and the way it integrates into the genome. **b.** Schematic illustration of the structure of the 3' trapping virus and the way it integrates into the genome. LTR, long terminal repeat; SA, splicing acceptor; SD, splicing donor; yellow box, bovine growth hormone polyA site; purple arrow, wild type *lox*P sites.

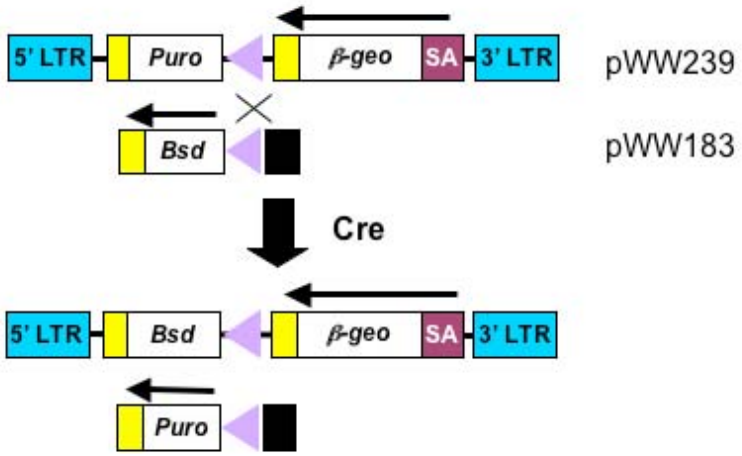


Fig. 3-14 Functional test of the 5' trapping virus. Schematic illustration of the outcome of Cre-mediated recombination between *PGK-lox*P-*Bsd-bpA* cassette and the promoter-less *lox*P-*Puro-bpA* cassette. LTR, long terminal repeat; SA, splicing acceptor; yellow box, bovine growth hormone polyA site; black box, *PGK* promoter; purple arrow, wild type *lox*P site.

3.2.2.2 3' trapping vectors

The original gene-trapping virus of Wentland et al. (unpublished data) contained a *PGK*-*Puro*-SD cassette. To improve the 3' trapping efficiency, different promoters were used to make a series of trapping vectors, and the trapping efficiency of different constructs was compared by *in vivo* functional test. pWW64 (*CAG* promoter), pWW65 (*EF1*α promoter) and pWW73 (*PGK* promoter) were linearized and then electroporated into AB2.2 ES cells. The transfectants were selected in M15 supplemented with puromycin. More puromycin resistant colonies were recovered from pWW64 than the other two plasmids, which suggested that the *CAG* promoter was much stronger than the other two promoters *in vivo* (Fig. 3-15). So the *CAG*-*Puro*-SD trapping cassette was used for future experiments.

The *CAG*-*Puro*-SD cassette and a promoter-less *lox*P-*Neo*-*bpA* cassette were cloned into pMSCV (Clontech) to make the final 3' trapping virus (pWW240), (Fig-3-13b). To test the function of the two cassettes, linearized pWW240 and pWW183 (*PGK*-*lox*P-*EM7*-*Bsd*-*bpA*) plasmids were co-electroporated into AB2.2 ES cells with or without a Cre expression plasmid, and the transfectants were selected with M15+G418, M15+blasticidin or M15+puromycin, respectively. As a control, linearized pWW240 plasmid was also electroporated into AB2.2 cells and these cells were selected with M15+G418 or M15+puromycin, respectively.

Puromycin resistant colonies were recovered when linearized pWW240 was electroporated into AB2.2 ES cell alone. This result confirmed the function of the 3' trapping cassette of the virus. No G418 resistant colonies were recovered when pWW240 was electroporated into AB2.2 ES cell alone. This result confirmed that promoter-less *lox*P-*Neo*-*bpA* cassette of the virus does not function. G418 resistant colonies were recovered when linearized pWW240 and pWW183 (*PGK*-*lox*P-*EM7*-*Bsd*-*bpA*) plasmid was co-electroporated into AB2.2 ES cells with a Cre expression plasmid. This result confirmed that when the non-functional *Neo* cassette gains the *PGK* promoter by Cre-mediated recombination, it becomes functional. This test has

confirmed that both halves of the virus were functional *in vivo* (Fig 3-16). This 3' trapping virus construct was used for future experiment.

For the same reason as 5' trapping, electroporation-based 3' trapping vector (pWW238) was also constructed. The two cassettes in pWW238 are the same as pWW240, but instead of cloning these two cassettes into a virus backbone, they were cloned into pBS vector. pWW238 was also tested in the same way as the pWW240. The results confirmed that both the trapping and inversion cassettes worked *in vivo*.

3.2.3 Retrovirus transfection

The gene-trap retroviral vector was used to transiently transfect the Phoenix (REF) viral packaging cells using the Calcium Phosphate method. In brief, Phoenix cells were transfected with supercoiled pWW239 DNA. Viral supernatant was harvested at different time points, combined together and filtered through 0.45 μm filters to remove the Phoenix cells in viral supernatant. 1 ml of viral supernatant was used to infect wild-type AB2.2 ES cells. The infected ES cells were selected in M15 supplemented with G418. The G418 resistant colonies were stained with Methylene Blue (Fig. 3-17a). The trapping titre for the 5' trapping virus was around 10 trapping events/ml virus supernatant.

Phoenix cells were transiently transfected with pWW240 DNA to package the 3' trapping retrovirus. Viral supernatant was harvested and 1 ml of viral supernatant was used to infect wild-type AB2.2 ES cells. The infected ES cells were selected in M15 supplemented with puromycin, and puromycin resistant colonies were stained with Methylene Blue (Fig. 3-17b). The trapping titer for the 5' trapping virus was around 5 trapping events/ml virus supernatant. Because the trapping titer was slightly lower than the 5' trapping virus and a portion of the insertions might represent trapping of cryptic splice acceptors and polyA signals, we decided to use the 5' trapping strategy.

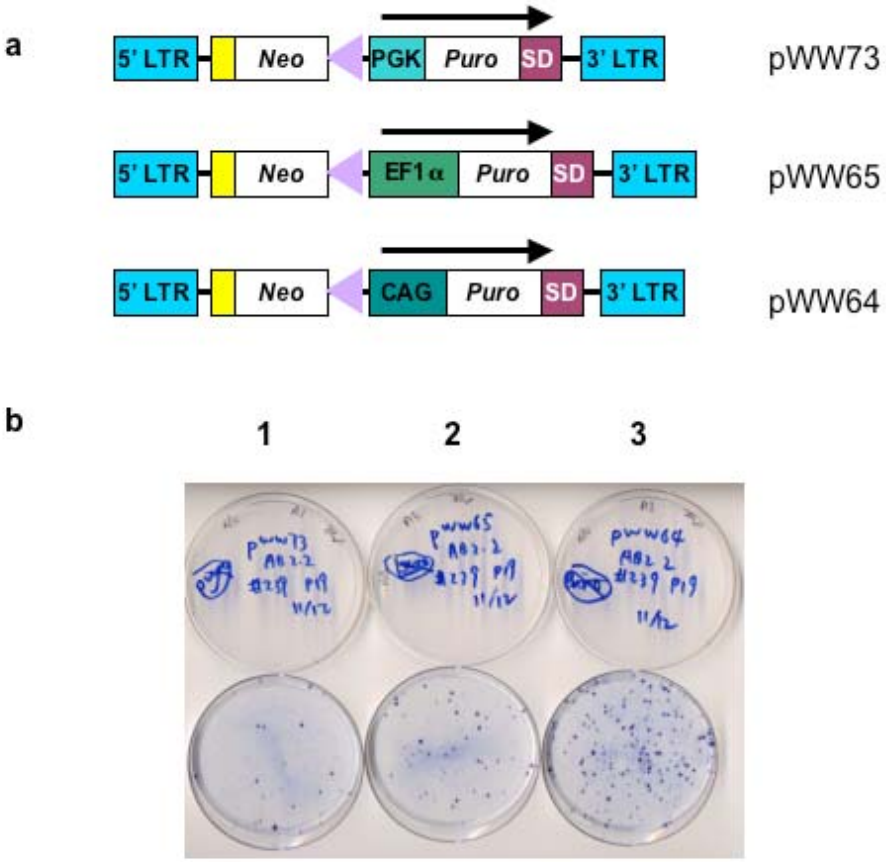


Fig. 3-15 Promoter test of the 3' trapping virus. a. Schematic illustration of the 3' trapping viruses sing different promoters. LTR, long terminal repeat; SD, splicing donor; yellow box, bovine growth hormone polyA site; purple arrow, wild type *lox*P site. **b.** Functional test of the 3' trapping virus construct.1: pWW73 (PGK promoter); 2: pWW65 (EF1α promoter); 3: pWW64 (CAG promoter). 20 μg of linearized pWW73, pWW64 and pWW65 was electroporated into AB2.2 ES cells, and the transfectants were selected with M15+puromycin.

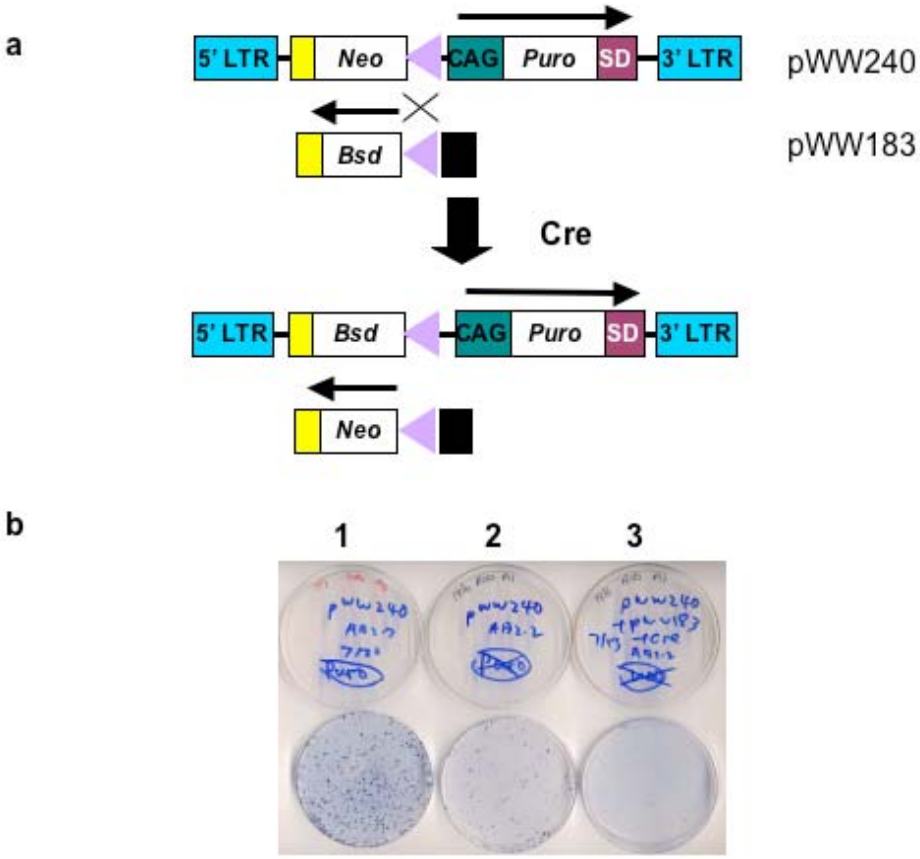


Fig. 3-16 Functional test of the 3' trapping virus. a. Schematic illustration of the outcome of the Cre-mediated recombination between the *PGK-lox*P-*Bsd-bpA* cassette and the promoter-less *lox*P-*Neo-bpA* cassette. LTR, long terminal repeat; SD, splicing donor; yellow box, bovine growth hormone polyA site; purple arrow, wild type *lox*P site. **b.** Functional test of the 5' trapping virus construct, pWW240. 1and 2: Linearized pWW240 was electroporated into AB2.2 ES cells and selected with M15+puromycin. Puromycin resistant colonies were recovered as expected, which confirm the function of the trapping cassette. 3: Linearized pWW240 and pWW183 plasmid DNA were co-electroporated into AB2.2 ES cells with the Cre expression plasmid which were selected with M15+G418. G418 resistant colonies were recovered as expected, which confirm the function of the inversion cassette.

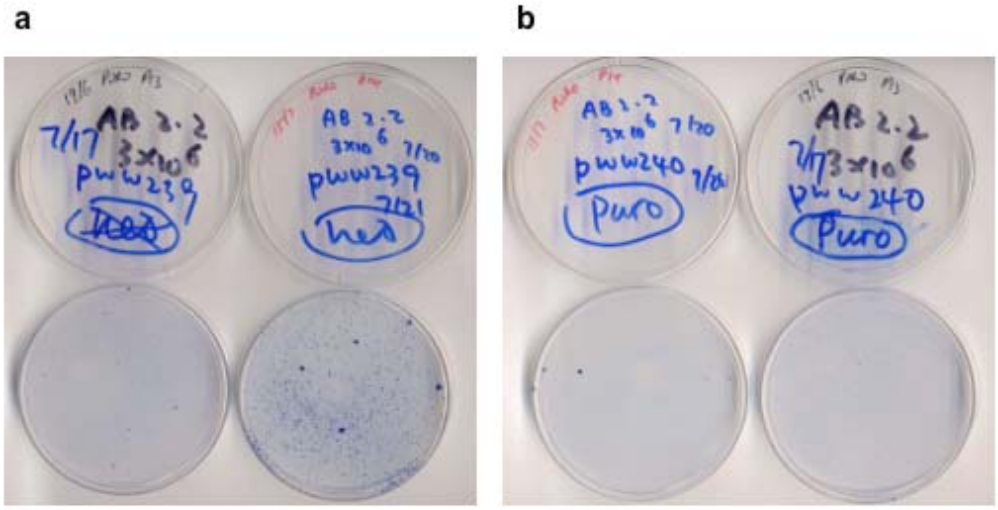


Fig. 3-17 The trapping titer of the 5' and 3' trapping retrovirus. a. 5' trapping retrovirus. Phoenix cells transfected with pWW239 DNA were used to make the 5' trapping retrovirus. Viral supernatant was harvested at different time points, combined together and filtered through 0.45 μm filters to get rid of the Phoenix cells. 1 ml of viral supernatant was used to infect the wild type AB2.2 ES cells. The infected ES cells were selected in M15 supplemented with G418. The G418 resistant colonies were stained with Methylene Blue. The virus infection experiment was repeated twice. 7 and 5 colonies were found on the two plates. Note that in the second experiment, G418 selection was released after 7 days. Many background colonies were found. So in the large-scale experiment, all the G418 colonies were selected for 10 days to reduce the background. **b.** 3' trapping retrovirus. Phoenix cells transfected with pWW240 DNA were used to make 3' trapping retrovirus. Viral supernatant was harvested at different time point,s combined together and filtered through 0.45 μm filters. 1 ml of viral supernatant was used to infect the wild type AB2.2 ES cells. The infected ES cells were selected in M15 supplemented with puromycin. The puromycin resistant colonies were stained with Methylene Blue. The virus infection experiment was repeated twice. 5 and 4 colonies were found on the two plates.

3.2.4 Pilot experiment to test the regional trapping strategy

3.2.4.1 Pilot experiment to test the intactness of proviral insertion

As a pilot experiment, the retrovirus supernatant was also used to infect WW69-C6 cells. Some G418 resistant colonies were randomly picked and expanded. Genomic DNA was extracted from these clones for Southern analysis (Fig. 3-18a). Since there is a single *Eco*RI site in SA-β*geo* cassette, individual virus integration events can be discriminated by the size of their unique proviral/host junction fragments, which are determined by the location of the endogenous *Eco*RI site nearest to the 3' LTR (Fig. 3-18b). There are two *Kpn*I sites in the 5' LTR and 3' LTRs of the retrovirus, so *Kpn*I digestion of the genomic DNA and subsequent hybridization using a *LacZ* probe should detect a 6.9 kb *Kpn*I restriction fragment from the intact provirus (Fig. 3-18c).

The Southern hybridization did identify a 6.9 kb *Kpn*I fragment for some trapped clones, but for the others, the hybridization detected an unexpected 6.0 kb fragment. A possible explanation for this is that an alternative splicing event happened in the transcription process when the retrovirus was replicated in the Phoenix cells. As the result, a part of the retrovirus would be skipped as an intron. If the alternative splicing event had occurred in the trapping or inversion cassettes, it would affect the trapping or the subsequent inversion. But if the splicing had occurred in a non-essential region in the virus backbone, it would not have any effect on the following steps.

Since the 6.0 kb *Kpn*I fragment was detected in the G418 resistant clones, the alternative splicing did not inactivate the trapping cassette. To determine whether the inversion cassette had been inactivated, the G418 clones were expanded and a Cre expression plasmid was electrorated into the trapped clones. The recombinants were selected in M15 supplemented with puromycin. Puromycin resistant colonies were recovered from the clones with the 6.0 kb *Kpn*I fragment, as well as the clones with the 6.9 kb fragment. So the alternative splicing event must have occurred somewhere on the virus backbone though this did not interfere with virus packaging and integration.

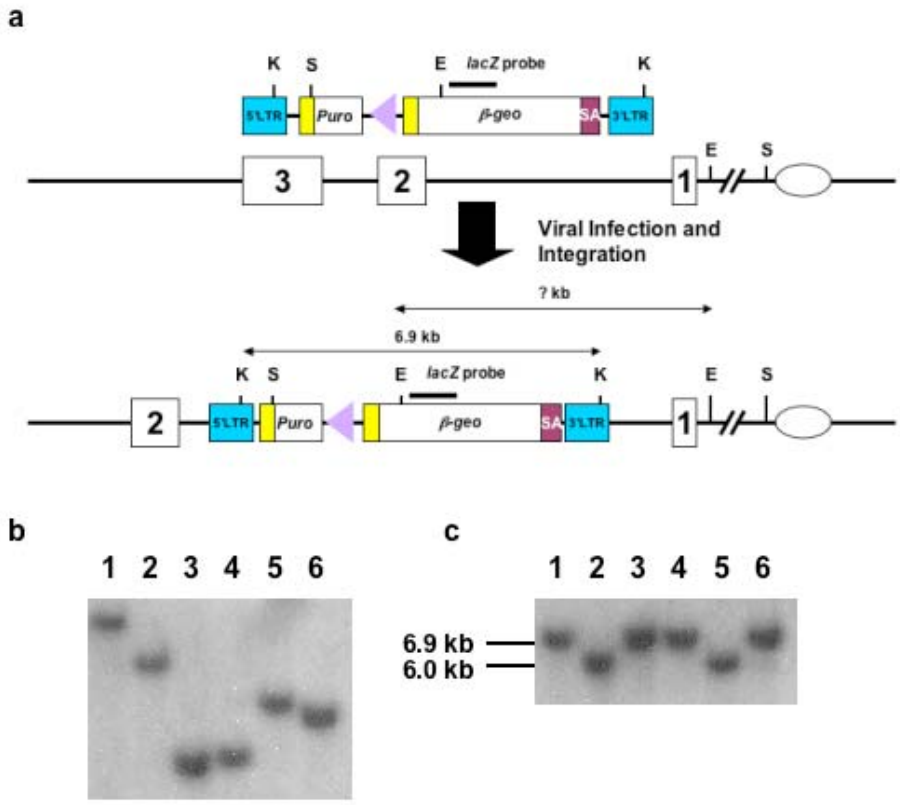
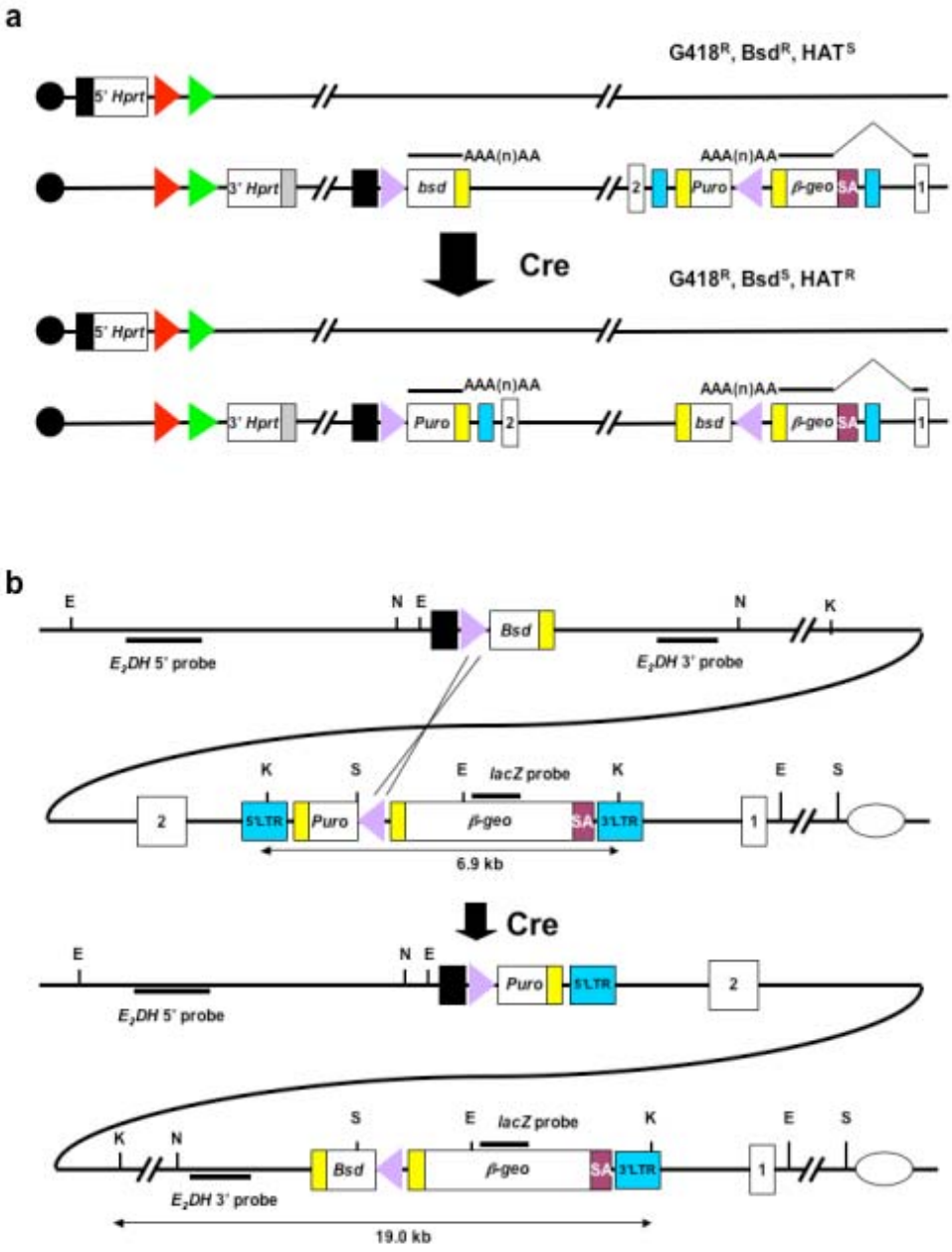


Fig. 3-18 Identification of individual virus integration events. a. Schematic illustration of retrovirus integration. LTR, long terminal repeat; black arrow, wild type *lox*P site; K, *Kpn*I, E, *Eco*RI. **b.** Genomic DNA from G418 resistant gene-trap clones were cut with *Eco*RI and hybridized to a *lacZ* probe. Individual insertion events will have unique proviral junction fragments, and these can be distinguished by different sizes of the restriction fragments. **c.** Genomic DNA from G418 resistant gene-trap clones cut with *Kpn*I and hybridized to a *lacZ* probe. A 6.9 kb intact proviral fragment was found for some of the clones. But for the others, an unexpected 6.0 fragment was found, which might result from alternative splicing during retrovirus replication process.

3.2.4.2 Pilot experiment to test the Cre-mediated inversion

Mouse chromosome 11 contains 1797 known or predicted genes, which consist 6.4% of all the 28069 mouse genes (NCBI m33 mouse assembly, freeze May 27, 2004, strain C57BL/6J), so there is a 6.4% chance that a gene-trap will occur on chromosome 11. Half of gene traps on chromosome 11 (3.2% of the total) are expected to occur on the end point cassette targeted homolog of chromosome 11. Half of the gene traps on the targeted homolog (1.6% of the total) will be in the correct orientation for an inversion. One-third of the gene traps (0.5% of the total) will be in the vicinity of the end point (*E_2DH*) for an inversion event to happen efficiently. Thus a strong selection strategy is needed to select for these rare events. In my project, a split promoter and selection marker was used to achieve efficient recovery of the inversion events (Fig. 3-19a).

To test whether the selection strategy works, a pilot experiment was carried out. The 5' gene-trap retrovirus was used to infect WW69-A12 ES cells, and 100 G418 resistant gene-trap clones were picked and pooled together. A Cre expression plasmid was electroporated into the pool to induce the inversion. The cells were selected in M15 supplemented with puromycin and 16 puromycin resistant colonies were randomly picked and expanded. Genomic DNA was extracted from these clones for Southern analysis (Fig. 3-19b). Genomic DNA was digested with *Kpn*I and hybridized with a *lacZ* probe to identify the inversion events. When a Cre-mediated inversion occurs, the 5' region of the *E_2DH* end point targeting cassettes will move adjacent to the 3' region of the retrovirus. In such puromycin resistant clones, the 6.9 kb or 6.0 kb proviral insertion fragment detected by the *lacZ* probe will be replaced by a 19.0 kb inversion fragment (Fig. 3-19c). Genomic DNA was also digested with *Eco*RI and hybridized with a *lacZ* probe to identify independent gene-trap events (Fig. 3-19d). In this pilot experiment, the puromycin resistant clones had a limited repertoire of proviral junction fragments; it is likely that clones with the same-sized *Eco*RI fragments are derived from the same gene-trap clone.


a
G418R, BsdR, HATS
5' Hprt
3' Hprt
bsd
AAA(n)AA
2
Puro
β-geo
SA
1
Cre
G418R, BsdS, HATR
b
E
N
Bsd
K
E2DH 5' probe
E2DH 3' probe
S
lacZ probe
5'LTR
3'LTR
6.9 kb
19.0 kb

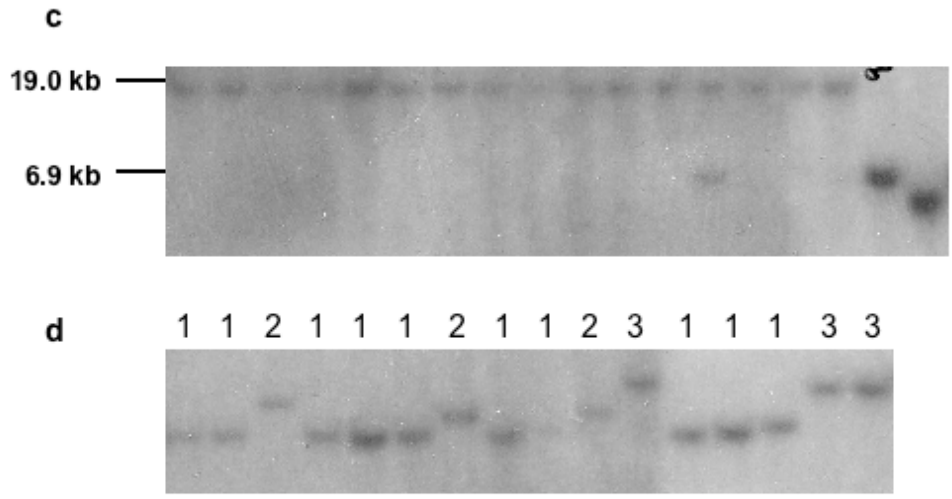


Fig. 3-19 Identification of individual inversion events. a. Schematic illustration of regional trapping induced by Cre transient expression. Black box: *PGK* promoter, yellow box: a bovine growth hormone polyA site; grey box: SV40 polyA site; blue box, long terminal repeat; SA, splicing acceptor; arrows of different color, variant *lox* sites. **b.** Schematic illustration of Southern screening strategy to identify individual inversion events. LTR, long terminal repeat; black arrow, wild type *lox*P site; K, *Kpn*I; E, *Eco*RI; N, *Nde*I; S, *Spe*I. **c.** Genomic DNA from Puromycin resistant clones was cut with *Kpn*I and hybridized to a *lacZ* probe. A 19.0 kb inversion fragment was detected. Note that one of the clones has both the 6.9 kb proviral insertion fragment and the 19.0 kb inversion fragment. The last two lanes are gene trap clones used as control. **d.** Genomic DNA from puromycin resistant gene-trap clones was cut with *Eco*RI and hybridized to a *lacZ* probe. Individual events will have unique proviral junction fragments, which can be resolved by different sized restriction fragments. Note that several clones share the same sized proviral junction fragments (group 1, 2 and 3), these are probably derived from the same trapping clone.

Clones that have rare proviral junction fragments might harbour inefficient recombination events, such as a balanced translocation, a balanced deletion/duplication, or an inversion over a long genetic distance. The balanced translocation and deletion/duplication do not involve loss of any genetic material, so these cells are expected to be viable at this step. Such clones can not become homozygous by induced mitotic recombination because this will result in the loss of a significant part of the chromosome 11 (Fig. 3-20).

3.2.5 Large-scale regional trapping experiment using gene-trap retrovirus

To perform large-scale experiments, 2000 ml of viral supernatant was harvested from 20X 90-mm plates of pWW239-transfected Phoenix cells. The viral supernatant was used to infect WW69-D6 ES cells plated on a total of twenty 90-mm feeder plates. Fresh viral supernatant was used to replace the old one every 12 hours for 3 days to increase the chance of viral infection. The gene-trap clones were then selected in M15 medium supplemented with G418. One plate was stained with Methylene Blue to count the number of the G418 resistant colonies. About 500 G418 resistant colonies were found on this plate. By extrapolation, there are around 10,000 independent gene-trap clones in the gene-trap library. The G418 resistant ES cell colonies from the remaining 19 retrovirus-infected plates were maintained as 19 subpools (WW99-1 to 19).

A Cre expression plasmid was electroporated into the subpools of gene-trap clones, WW99-1 to 19. The recombinants were selected in M15 supplemented with puromycin. Most of the plates had more than 100 puromycin resistant colonies. Some plates (WW103-RT-3, 10, 17 and 19) had less than 100 colonies, while WW103-RT-16 only had about 10 puromycin resistant colonies. The variation in puromycin resistant colony number from plate to plate might represent the variation in the proportion and position of gene-traps on the right chromosome. The puromycin resistant ES cell colonies were pooled together to make WW103-RT-1 to 19.

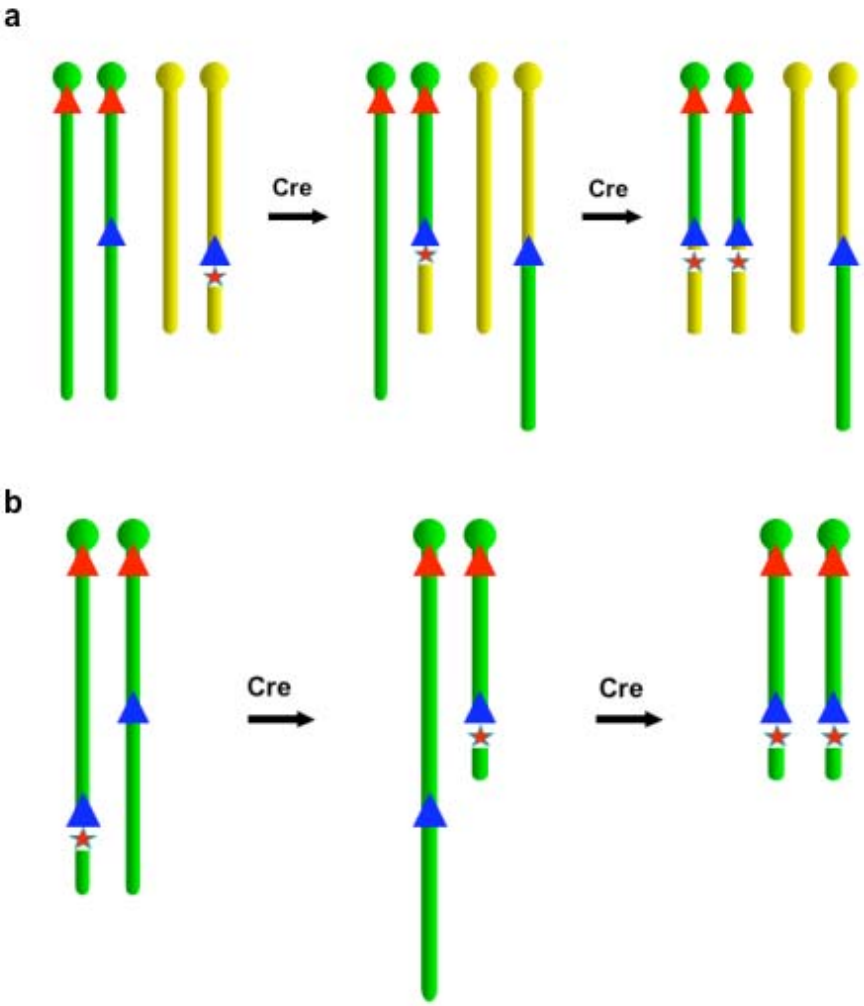


Fig. 3-20 Balanced translocation and deletion/duplication events can not be made homozygous. a. Balanced translocation events cannot become homozygous because this will cause loss of one copy of the distal part of chromosome 11. **b.** Balanced deletion/duplication events cannot become homozygous because this will cause loss of both copies of the distal part of chromosome 11. Red arrow, mutant *lox* sites used for induced mitotic recombination; blue arrow, wild type loxP sites used for regional trapping; red star, gene-trap mutation; green and yellow bar, different chromosomes.

3.2.6 Regional trapping experiment using electroporation-based plasmid

Linearized pWW237 plasmid DNA was electroporated into WW69-C6 ES cells. The recombinants were plated on 10X 90-mm feeder plates and selected in M15 supplemented with G418. The G418 resistant colonies were maintained as 10 subpools (WW100-1 to 10).

Cre expression plasmid was electroporated into the subpools of plasmid-based gene-trap clones, WW100-1 to 10. The recombinants were selected in M15 supplemented with puromycin. The puromycin resistant ES cell colonies were pooled together to make WW104-1 to 10.

3.2.7 Induced mitotic recombination

Both the induced mitotic recombination and the regional trapping are mediated by the Cre/*lox*P system. The Inducible mitotic recombination cassettes contain the mutant *lox* sites, *lox*5171 and *lox*2272, and the regional trapping cassettes contain the wild-type *lox*P site. These variant *lox* sites were used because they can efficiently recombine with themselves but not with each other. This reduces the chance that the two events (inversion and mitotic recombination) will interfere with each other.

However, there is possibility that the mitotic recombination and the inversion events can happen simultaneously at the first round of Cre expression, which will produce HAT and puromycin double resistant clones. In such a circumstance, when the puromycin resistant colonies are pooled, every single cell from the double resistant colonies will be able to survive the HAT selection. If this happens, these cells will dominate the pool and it will be almost impossible to identify other HAT resistant clones from the same pool after mitotic recombination induced by the second round of Cre-mediated recombination.

The induced mitotic recombination rate at the *D11Mit71* locus, which I used to create the induced mitotic recombination cell line, is $3.5 \pm 1.8 \times 10^{-4}$/cell

electroporated (Liu, Jenkins et al. 2002). And the efficiency of Cre recombination over a physical distance of 34 Mb (*Wnt3-p53*) is 2.2 ± 0.6 X 10^{-3}/cell electroporated (Zheng, Sage et al. 2000). So it is reasonable to predict that the chance of the two events happening simultaneously is very low.

In the large-scale experiments, Cre expression plasmid was electroporated into the pools of inversion clones, WW103-RT-1 to 19. The recombinants were selected in M15 supplemented with HAT. Most of the plates had around 1000 HAT resistant colonies, which is comparable with the colony number obtained from the parent cell line WW69-C6 after Cre transient expression. But for the pools WW103-RT-1 and 15, the cell density was too high to form single colonies. So no colonies were picked for these two plates. It is possible that for these pools, prior to the second round of Cre-mediated recombination, some clones were already HAT resistant. For the remaining plates, 48 colonies were randomly picked and cultured on 96-well feeder plates.

Genomic DNA was extracted from the 96-well plates and Southern analysis was carried out to determine the genotype of every HAT resistant clone. Because the inversions become homozygous when mitotic recombination is induced by Cre expression, both end points of the inversions are homozygous for the targeted alleles (Fig. 3-21). It is impossible to identify the genotype of the trapped loci without knowing the proviral integration sites. It is not feasible to carry out 1,000 inverse PCR or splinkerette PCR to identify the trapped locus for every HAT resistant clone and design allele specific probes to verify the genotypes of each one of them. So the genotype of the *E_2DH* locus was used to determine the genotype of the other ends of the inversions.

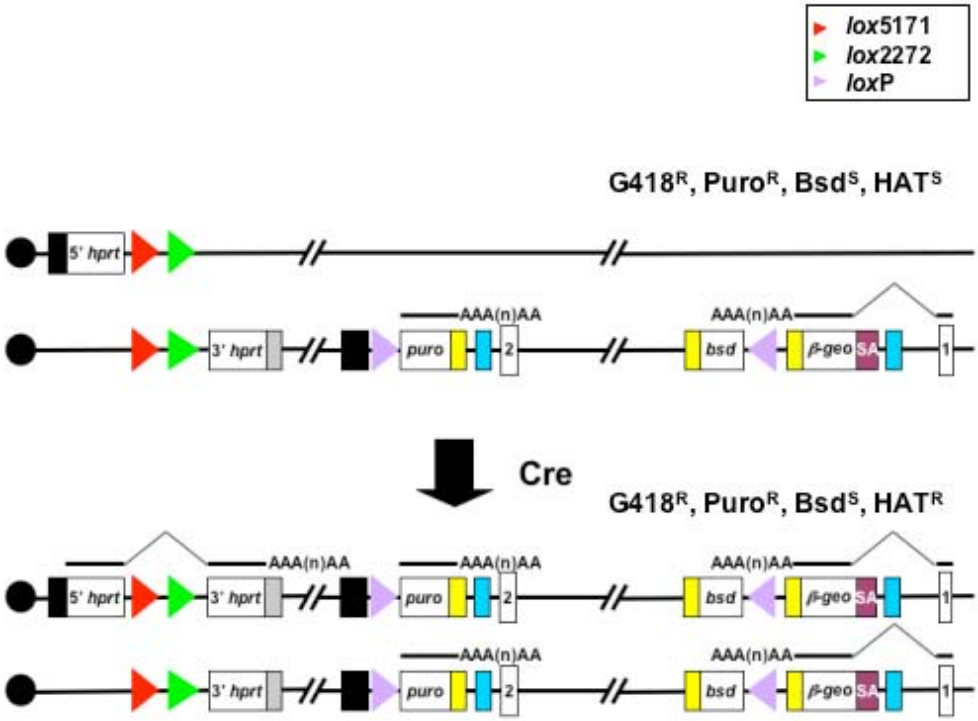


Fig. 3-21 Induced mitotic recombination to make the inversions homozygous. Schematic illustration of mitotic recombination to make the inversions homozygous. Black box, *PGK* promoter; yellow box, bovine growth hormone polyA site; grey box: SV40 polyA site; blue box, long terminal repeat; SA, splicing acceptor; arrows of different colours, variant *lox* sites. Note that the targeted E_2DH locus also becomes homozygous after induced mitotic recombination. So the genotype of the E_2DH locus can be used to represent the genotypes of the other ends of the inversions.

Individual recombination events were identified by their unique proviral/host junction fragments generated by *Eco*RI and *Spe*I digestion (*Eco*RI and *Spe*I are unique sites in the virus). If two clones from a same pool exhibit the same-sized proviral junctions fragments by two different restriction enzyme digestions (*Eco*RI and *Spe*I), they were considered as daughter clones from the same gene-trap and recombination event, and were therefore grouped together.

For most pools, homozygous clones were identified by Southern analysis using an E_2DH 3' probe (Fig. 3-22a and b). But from pool WW103-2, all of the HAT resistant clones are heterozygous and they all have the same-sized *Eco*RI proviral/host junction fragments (Fig. 3-22c and d). One possible explanation for this is that in this pool, a G2-Z event occurred at the same time as the inversion event, which resulted into a HAT resistant, puromycin resistant heterozygous clone. This double resistant heterozygous clone would expand with the pool and these cells would be much more numerous than the other recombinants after the second Cre-mediated recombination event. Homozygous clones were recovered from the other 16 pools (WW103-3 to WW103-14, WW103-16 to WW103-19).

The homozygous clones from the 16 plates were classified according to the sizes of their proviral junction fragments. For the groups that were represented by more than one clone, at least 2 independent clones were expanded. For the groups that had only one clone, the clone was expanded. Genomic DNA and RNA were extracted from all of the expanded clones. Southern analysis was carried out using different probes and enzyme digestions to confirm their genotypes (Fig. 3-23).

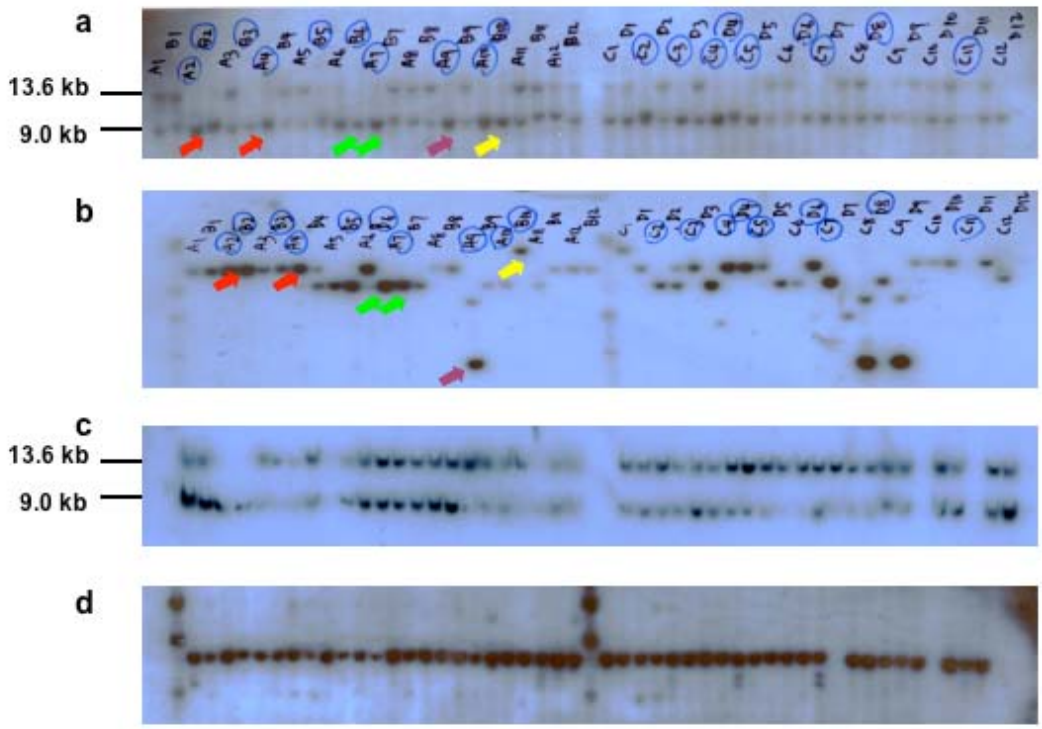


Fig. 3-22 Identification of the induced mitotic recombination clones. **a.** Mini-Southern analysis of clones from WW103-7. Genomic DNA was digested with *Nde*I and hybridized with the E_2DH 3' probe. Homozygous clones only have the targeted restriction fragment of 9.6 kb, and lack the wild type restriction fragment (13.1 kb). **b.** Mini-Southern analysis of clones from WW103-7. Genomic DNA was digested with *Eco*RI and hybridized with the *lacZ* probe. Individual recombination events were grouped according to their unique proviral junction fragments. Arrows of the same colour identify clones with the same sized *Eco*RI junction fragments. **c.** Mini-Southern analysis of clones from WW103-2. Genomic DNA was digested with *Nde*I and hybridized with the E_2DH 3' probe. No homozygous clones were identified. **d.** Mini-Southern analysis of clones from WW103-2. Genomic DNA was digested with *Eco*RI digestion and hybridized with the *lacZ* probe. All the clones showed the same sized *Eco*RI junction fragments.

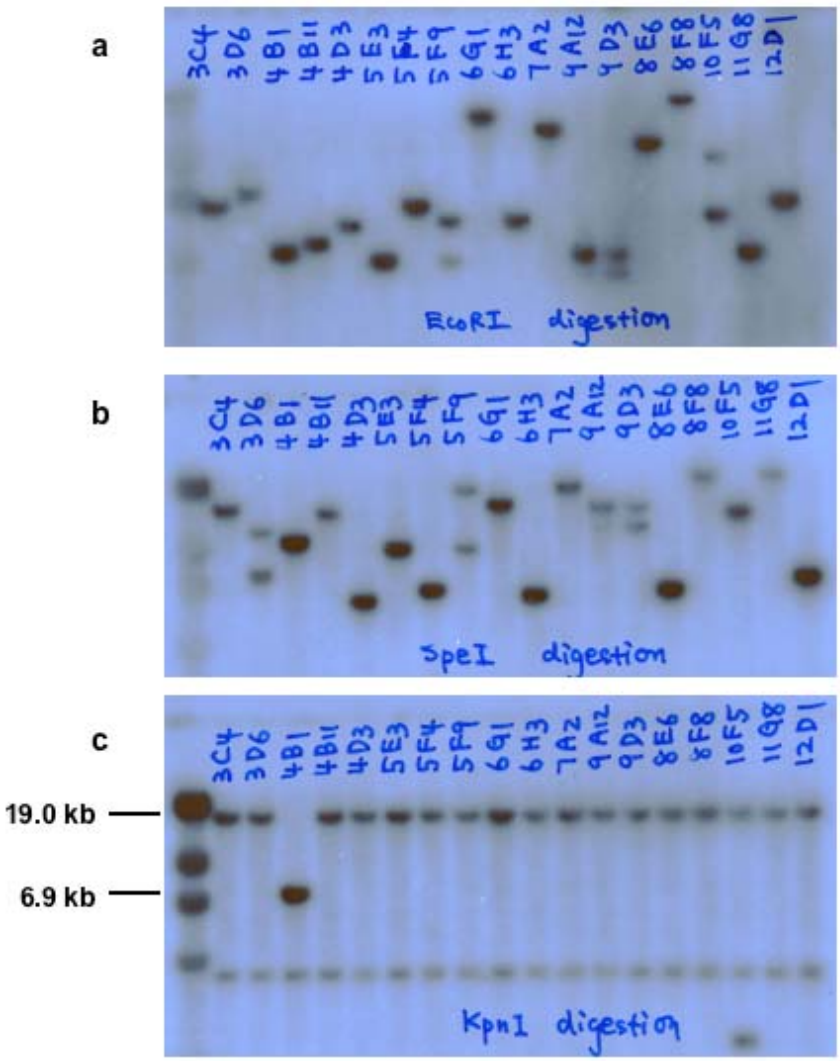


Fig. 3-23 Confimation of the homozygous clones. a. Genomic DNA extracted from WW103 homozygous clones was digested with *Eco*RI and hybridized with a *lacZ* probe. **b.** Genomic DNA from WW103 homozygous clones was digested with *Spe*I and hybridized with a *lacZ* probe. **c.** Genomic DNA from WW103 homozygous clones was digested with *Kpn*I and hybridized with a *lacZ* probe. Note that WW103-4B1 has only 6.9 kb proviral insertion fragment , and all the other clones only have the 19.0 kb inversion fragment .

Most of the clones only have a 19 kb *Kpn*I inversion fragment detected by the lacZ probe, some clones have only the 6.9 kb proviral insertion fragment, while the rest will have both. Sometime, these three different genotypes were found in clones of the same group. The explanation for this is that expression of Cre to induce mitotic recombination will in some case revert the inversion, especially the small ones. Depending on the sequence of these two events, the resulting clones can carry two gene-trap alleles or one inversion allele and one gene-trap allele (Fig. 3-24). These clones with reverted inversions can not be distinguished from other inversion clones by drug selection because they still have one functional *Puro* selection marker. A total of 146 clones, which could be classified into 66 independent groups, were expanded and DNA and RNA samples were taken to identify the virus integration sites and the trapped exons.

Cre expression plasmid was also electroporated into pools WW104-1 to 10. The recombinants were selected in M15 supplemented with HAT. 48 HAT resistant colonies were picked and Southern analysis was carried out to genotype the clones using the same strategy described before for the WW103 pools. The *E_2DH* 3' probe successfully identified homozygous inversions. However, the *lacZ* probe failed to identify unique junction fragments. Irrespective of which restriction enzyme was used, multiple bands were detected for almost all the clones. It was therefore difficult to group the clones according to their digestion pattern. In principle, 5' RACE followed by sequence analysis could be used to identify the trapping exons of all the homozygous clones. But this approach is very labour-intensive, so no further characterization was carried out for the clones generated by plasmid-based gene-trap strategy.

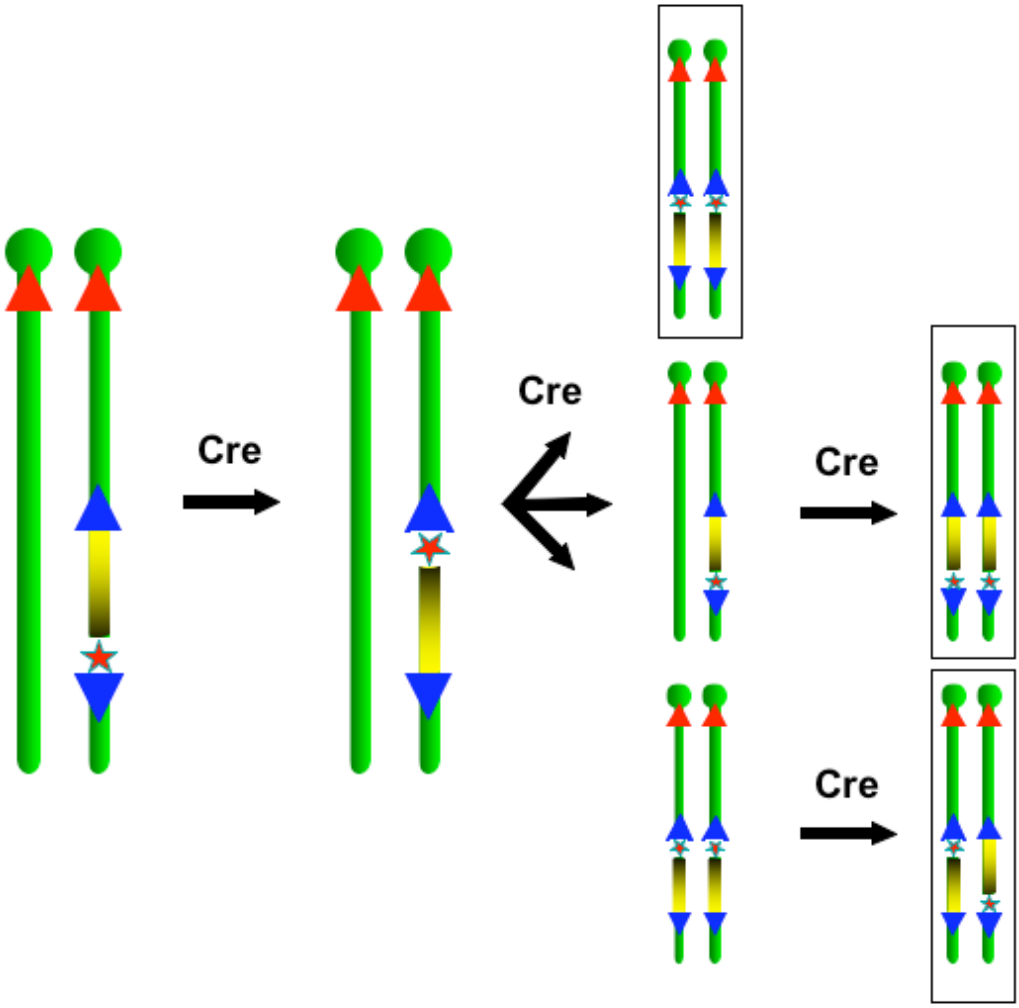


Fig. 3-24 Revertible inversion. The regional inversion can be inverted back with transient expression of Cre. The efficiency of the reverse inversion will be extremely high if the original inversion happens over a short physical distance. Depending on the sequence of induced mitotic recombination and the reverse inversion, the resulting homologous clones can carry either two trapping alleles or one trapping allele and one inversion allele. Red arrow, mutation *lox* sites used for induced mitotic recombination; blue arrow, wild loxP sites used for regional trapping; red star, gene-trap mutations; green bar, chromosome 11; yellow bar, the inversion region.

3.3 Discussion

As discussed in the previous chapters, homozygous mutant mouse ES cells are a very important resource for functional studies *in vitro*. The existing methods to generate homozygous clones require designing and constructing targeting and genotyping strategies for each different gene, which is difficult to scale up. This has greatly limited the effort to utilise homozygous mutant ES cells for genetic screens.

We have designed a strategy to circumvent this bottleneck. Heterozygous mutations were generated by regional trapping, and these mutations were converted to homozygosity by induced mitotic recombination. Strong selection strategies, a split *Hprt* minigene and a split *PGK*/*Puro*, were chosen to recover these rare events. Variant *lox* sites were used to avoid interference between the two separate selection systems.

For regional trapping, the first *lox*P site was targeted to the E_2DH locus, and the second *lox*P site was introduced into the genome by a retroviral vector. As the result of random integration of the retrovirus, the direction of the two *lox*P sites can be either the same or opposite. The *lox*P sites can also be located on the same chromosome (*cis*) or on different ones (*trans*). The outcome of the recombination event is directly determined by the location and the direction of the second *lox*P site (Fig. 3-25).

Of the four possible recombination products generated from a cell in G1 phase (G2 events will be discussed in the next chapter), inversion, balanced deletion/duplication and translocation do not result in loss of genetic material, and are therefore viable, unless the chromosomal breakpoints disrupt gene(s) that are essential for ES cell self-renewal. Dicentric/acentric chromosomes are not viable. For the deletion, the viability of the resulting recombinant depends on the size of the deletion and the genes in the deletion region. In the nested deletion experiment (Su, Wang et al. 2000), most of the deletions recovered were mapped within 1 cM distal or proximal to the anchor point. It is possible that deletions larger than that will cause haplosufficiency. Other efforts to generate large deletions also encountered the same problem (Liu,

Zhang et al. 1998; Zheng, Sage et al. 2000). Although the recombination can still occur, the resulting recombinants sometimes duplicate the wild-type chromosome to compensate for the loss caused by the large deletion.

However, no homozygous deletion clones have been identified in my experiment. If the two *lox*P sites are in direct orientation on the same homolog of chromosome 11 (*cis*) and the trapped locus is proximal to *Hsd17b1,* the resulting deletion cells can not survive subsequent puromycin selection because the *Puro* cassette is deleted (Fig. 3-26a). If the two *lox*P sites are in direct orientation on the same homolog of chromosome 11 (*cis*) and the trapped locus is distal to *Hsd17b1*, the resulting deletion cells can survive subsequent puromycin selection. However, even if the heterozygous (before mitotic recombination) and homozygous (after mitotic recombination) deletions do not cause haplosufficiency or homozygous lethality in ES cells, these cells will not be identified by Southern because the β-*geo* cassette is deleted (Fig. 3-26b) and will therefore be discarded from the screen.

In a pilot experiment carried out to test the experimental design. 100 gene-trap clones were pooled and inversions were induced by Cre transient expression. From this experiment, puromycin resistant clones were successfully recovered and Southern analysis confirmed the recombination events. It is interesting to notice that most of the clones in this pilot had the same-sized proviral junction fragments. These clones are likely derived from a recombination event of high efficiency, most likely a small deletion or inversion. Those clones that have rare proviral junction fragments might represent inefficient recombination events, such as balanced translocations, balanced deletion/duplications, or large inversions.

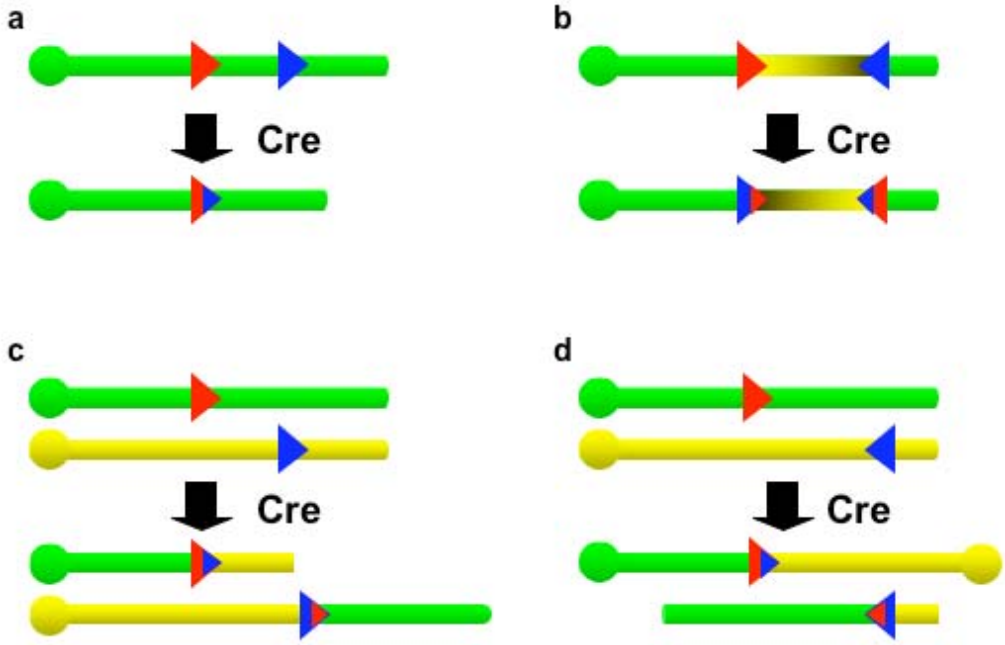


Fig. 3-25 Possible results of the Cre-mediated recombination (G1). a. *lox*P sites in direct orientation on the same chromosome (*cis*). A deletion will be generated by Cre-mediated recombination. The deletion might cause haplosufficiency depending on the size of the deletion. **b.** *lox*P sites in inverted orientation on the same chromosome (*cis*). An inversion will be generated by Cre-mediated recombination. **c.** *lox*P sites in direct orientation on different chromosomes (*trans*). A balanced deletion/duplication will be generated by Cre-mediated recombination. **d.** *lox*P sites in inverted orientation on different chromosomes (*trans*). A deletion will be create by Cre-mediated recombination. A dicentric and an acentric chromosome will be generated by Cre-mediation recombination, and the resulting cells are not viable. Red arrow,end point *lox*P site; blue arrow, *lox*P site introduced by retrovirus.

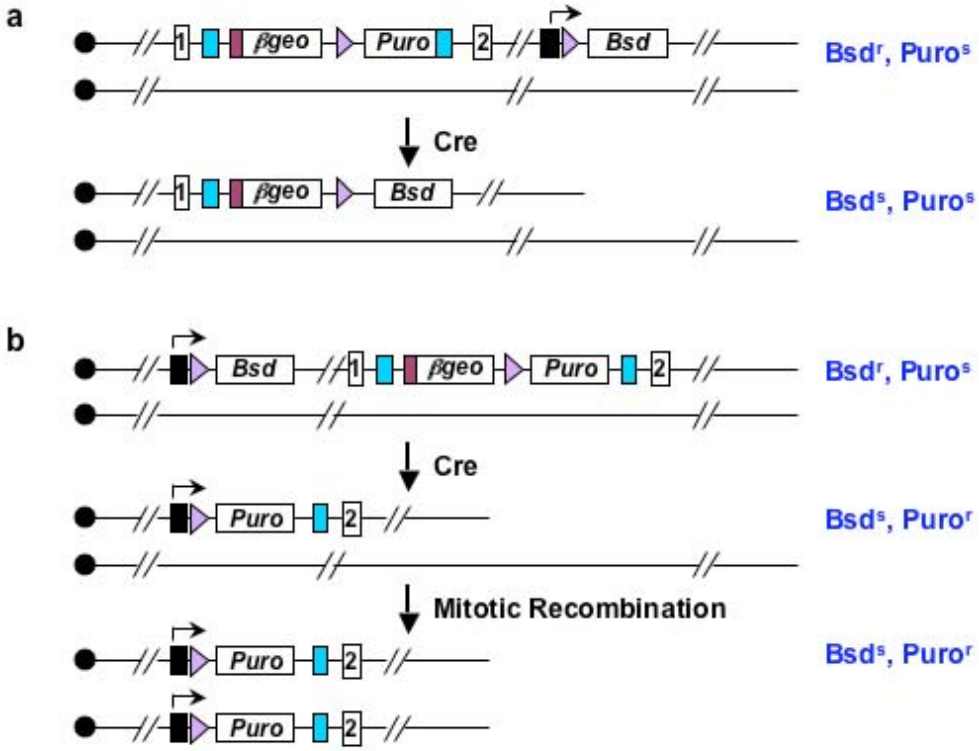


Fig. 3-26 Deletion. ***a.*** *lox*P sites in direct orientation on the same homolog of chromosome 11 (*cis*) and the trapped locus is proximal to *Hsd17b1*. A deletion will be generated by Cre-mediated recombination. The resulting cells can not survive subsequent puromycin selection because the *Puro* cassette is deleted. ***b.*** *lox*P sites in direct orientation on the same homolog of chromosome 11 (*cis*) and the trapped locus is distal to *Hsd17b1*. A deletion will be generated by Cre-mediated recombination. The resulting cells can survive subsequent puromycin selection. However, even if the heterozygous (before mitotic recombination) and homozygous (after mitotic recombination) deletions do not cause haplosufficiency or homozygous lethality in ES cells, they will not be identified by Southern because the *β-geo* cassette is deleted. Black box, *PGK* promoter; lavender triangle, wild-type *lox*P site; blue box, virus long terminal repeat; plum box, splice acceptor.

A balanced translocation or deletion/duplication will not result in loss of genetic material, and therefore should be recovered after the first Cre-mediated recombination and subsequent puromycin selection. However the Cre-induced mitotic recombination and HAT selection will select against those clones with translocations, because one copy of the distal part of chromosome 11 will be lost after mitotic recombination (Fig. 3-19). For a balanced deletion/duplication clone, depending on the position of the deletion chromosome, mitotic recombination will either result in a homozygous deletion (deletion occurs on the same chromosome as the 3' *Hprt* cassette) or a homozygous duplication clone (deletion occurs on the same chromosome as the 5' *Hprt* cassette). While the homozygous deletion clones are not viable, the homozygous duplication clones should survive because they do not loss any genetic material. This possibility will be discussed in more detail in the next chapter.

When the induced mitotic recombination clones from the same pool were analyzed by Southern, most of the homozygous clones exhibited a limited number of proviral junction fragments. It is likely that those clones carry very small inversions. The efficiency of generating small inversions is so high that most of the puromycin resistant colonies have arisen from one parental clone. Assuming such clones are viable after mitotic recombination, they will also dominate the population of HAT resistant colonies. The dominance of these small inversions has the potential to limit the coverage of the screen.

There are two ways to avoid this bias. First, more HAT resistant clones can be picked from each induced mitotic recombination pool for Southern analysis. Second, a smaller pool of trapped clones can be used for regional trapping (Wentland, unpublished data). By calculation, less than 1% the trapped clones will be on the right chromosome and in the right direction for an inversion. So in a pool of 100 trapped events, 0-1 inversion is expected after the Cre electroporation. If a pool yields many puromycin resistant colonies, these are likely to be the same inversion represented by many subclones. If just a few puromycin resistant colonies are recovered, they might reflect large inversion, deletion/duplication or translocation events. After induced mitotic

recombination, clones derived from the large inversion will dominate the pool of HAT resistant clones because they have a selection advantage over the translocations and deletion/duplications. The second method proved to work very well in Wentland's experiment. Inversions as big as 100 Mb were recovered (Wentland, unpublished data), but this method greatly increases the number of electroporation needed. For a total of 10,000 trapped clones, 100 electroporations are needed for regional trapping and another 100 electroporations for induced mitotic recombination. The extra effort can not guarantee the generation of 10 times more unique homozygous clones. So we decided to use a much larger pool of 500 trapped clones for the experiment. It is likely that most large inversions will be eliminated in the selection process.

Another issue of concern is that regional trapping and induced mitotic recombination can happen at the same time. Though variant *lox* sites were used to avoid interference between the two events, they can still take place simultaneously and generate puromycin and HAT double resistant clones after the first Cre-mediated recombination event. If this happens, the descendents of the double resistant colonies will dominate the newly formed HAT resistant colonies after the second Cre-mediated recombination. The use of Flp/*FRT* system is a possible alternative for one of the events. If Cre/*lox*P is used for regional trapping and Flp/*FRT* is used for induced mitotic recombination, then the two events can be separated. To test this system, an *FRT* site was inserted into multi-*lox* site linker (Fig. 3-2c) and a FPL expression plasmid, p*CAG*G-FLPe (Genbridge), was electroporated into the D11Mit71$^{5'\ Hprt\ /\ 3'\ Hprt}$ cell line, WW45. and recombinants were selected in M15 supplemented with HAT. Though HAT resistant colonies were recovered, the efficiency is about 2 to 3 orders of magnitude lower than Cre/*lox*P system (data not shown). So in the large-scale experiments, Cre was used for both regional trapping and induced mitotic recombination.

Homozygous inversions were identified from the HAT resistant clones from 16 out of 19 pools. For the other three pools, two (WW103-1 and 15) had too many HAT resistant colonies and one (WW103-2) was composed of the

heterozygous clones with the same proviral junction fragment. Most likely, in these pools, mitotic recombination was induced at about the same time as the inversion. Because the induced mitotic recombination will generate a functional *Hprt* mini-gene, it is possible to select against the double resistant colonies using 6-thioguanine (6-TG) after the first Cre mediated recombination because in cells with functional *Hprt* gene, 6-TG can be used to produce 2'-deoxy-6-thioguanosine-triphosphate, the active guanine nucleotide analogue in DNA synthesis, and thus kill the HAT^R cells.

Both 5' trapping and 3' trapping constructs were designed and tested. The titre of the 3' trapping virus is slightly lower than the 5' trapping virus. Considering that 3' trapping is more likely to trap cryptic splice acceptors and pseudo polyadenylation signals scattering throughout the genome, the mutagenicity of 3' trapping is not as high as 5' trapping. So we decided to choose 5' trapping strategy for the large-scale experiments. However, 3' trapping can trap genes that do not normally express in undifferentiated ES cells, which is an advantage for *in vitro* differentiation studies. For example, a gene required for mesoderm formation but not expressed in undifferentiated ES cells can only be mutated by 3' trapping strategy.

Both retroviral- and plasmid-based trapping constructs were designed and used in the large-scale experiments. The classification of trapped clones into groups according to their insertion/host junction fragments was straightforward for the clones generated from the virus, however the clones generated by electroporation of linearized trapping vectors always displayed multiple fragments which made them difficult to be classified by their Southern pattern. The multiple fragments detected can be caused by either concatermerization at a single insertion site or multiple insertions throughout the genome. Though conditions can be optimized to minimize the possibility of concatermerization, it will still occur in about 20% cells (Stanford, Cohn et al. 2001). Concatermerization can also result in ectopic reporter expression leading to expression of the reporter without trapping an endogenous gene. Also, the gene-trap vectors can be randomly truncated when they integrate into the genome. The differing lengths of the truncation make the cloning of

the flanking genomic sequence by Inverse PCR problematic. Since trapping by the retroviral vector generated enough clones for downstream analysis, the clones generated by electroporation have not been further characterized.

Southern analysis of the proviral junction fragments identified 66 different homozygous clones from a total of 16 plates of gene-trap clones. Each original trapping plate contains about 500 independent trapping events. So the proportion of homozygous gene-trap clones recovered from 8000 gene-trap events 0.75%. This efficiency is almost the same as we predicted before the experiment, thus the two selection strategies used to induce regional trapping and mitotic recombination are efficient enough to isolate these rare events.

In the chapter, I described the variables I tested and the strategy I chose to isolate homozygously mutated ES cell clones by regional trapping and induced mitotic recombination. Genotyping is not required for the trapping and inversion events. A common genotyping strategy was used at the final stage to genotype all the clones that underwent induced mitotic recombination. This has greatly simplified the genotyping procedure for identifying a large number of homozygous mutant clones. The different lengths of proviral/host flanking fragments were used to group the homozygous clones from the same pool. This procedure reduces the redundancy of the clones which is caused by the use of pooling to handle large numbers of clones. In turn, this reduces the number of clones that needed to be identified by 5’ RACE and splinkerette PCR. The results obtained proved that our strategy can efficiently isolated homozygous mutant clones without any previous knowledge of the loci that have been disrupted. This is an obvious advantage compared to traditional methods to generate homozygous mutant clones. Conditions can still be optimized to improve the yield. Therefore, I have shown that this strategy can be used to isolate homozygous mutant ES cell clones in an efficient way.