Chapter 5

Isolation of homozygous mutants in *Blm*-deficient ES cells based on copy number

5.1 Introduction

In this chapter I will describe preliminary experiments that I conducted to test the general method, and my construct in particular, for isolation of homozygous mutant ES cells. I created a library of single copy heterozygous mutations in *Blm* ES cells, and mapped the mutations by sequencing transposon-genome junction fragments. These clones were then used to test whether homozygous mutants could be recovered after expansion by selection based on the copy number of the transposon, which will be two in homozygous mutants but one in the heterozygous starting population. By conducting experiments on a small scale clone-by-clone basis I aimed to verify the mutagenicity and utility of my transposon construct, gain an understanding of how the loss of heterozygosity (LOH) process occurs, and potentially isolate some interesting mutants.

5.1.1 Copy number based selection

The method for isolation of homozygous mutations depends critically on a single copy insertion in the starting population of cells. As *Blm*-deficient cells with a heterozygous transposon mutation are expanded, they will segregate homozygous mutants at a low frequency as described earlier. These homozygotes will contain two copies of the transposon construct. The purpose of my transposon construct is to allow selective discrimination between cells with one and two copies. Cells with one copy will form the majority of the culture after expansion, with a minority of cells being homozygotes with two allelic copies that are "useful" for genetic screens. The culture will also contain cells that have lost the insertion and reverted to wild type as a consequence of the reciprocal LOH event that generates the homozygous mutants. As described in Chapter 3, the transposon construct contains a selection cassette encoding two mutually exclusively expressed resistant genes. Only homozygotes, which have two copies, are able to express both genes simultaneously after Cre recombinase treatment; these cells can therefore be selected in a combination of G418 and puromycin.

5.2 Results

5.2.1 Generation of single copy insertions

The experimental design is shown in Figure 5.1. For copy number selection to work it is important to limit the transposon to a single copy to begin with. This could be accomplished by mobilising the transposon from the single copy *Hprt* locus on the X chromosome. However, for simplicity in these experiments, I decided to generate the initial single copy clones by mobilisation of the transposon from a limiting quantity of plasmid coelectroporated with the transposase expression plasmid. This has been shown to result in mostly single copy insertions (Wang *et al.*, 2008). I used 100 ng of TNP transposon plasmid (i.e. *puro*-expressing construct, described in Chapter 3) with 10 μg pCMV-mPBase (Cadiñanos and Bradley, 2007) to transfect ten million NN5 ES cells in a volume of 0.9 ml. Cells were selected with puromycin for eight days and colonies picked. Analysis of the clones by Southern blot using a probe and restriction digest that allows discrimination of different insertion sites showed that the resulting colonies contained more than one insertion. However, the bands were clearly of different intensities, suggesting that the copy numbers of the corresponding insertions within the colony were different (Figure 5.2A, B). This could occur if the colony is in fact an unequal mixture of cells carrying different single copy insertions. To test this possibility, I repeated the process but replated the cells four days after transfection. Most subclones picked from this experiment bore single copy insertions (Figure 5.2C). The multiple bands seen in the first experiment likely arose from secondary genome to genome transposition events before the transposase activity was lost, resulting in mosaic clones. Another possibility is that two or more plasmid to genome transposition events occurred early in the growth of the colony, but after the founding cell had divided.

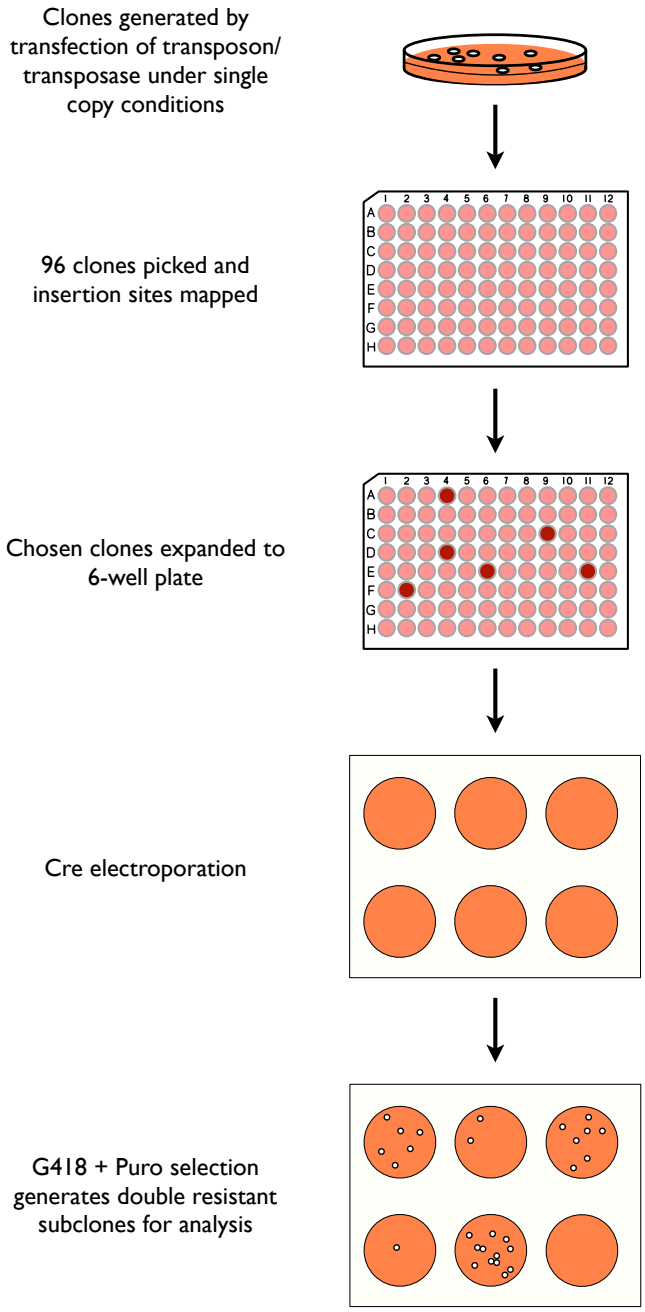


Figure 5.1: Experimental scheme for clone-by-clone isolation of homozygous mutants.

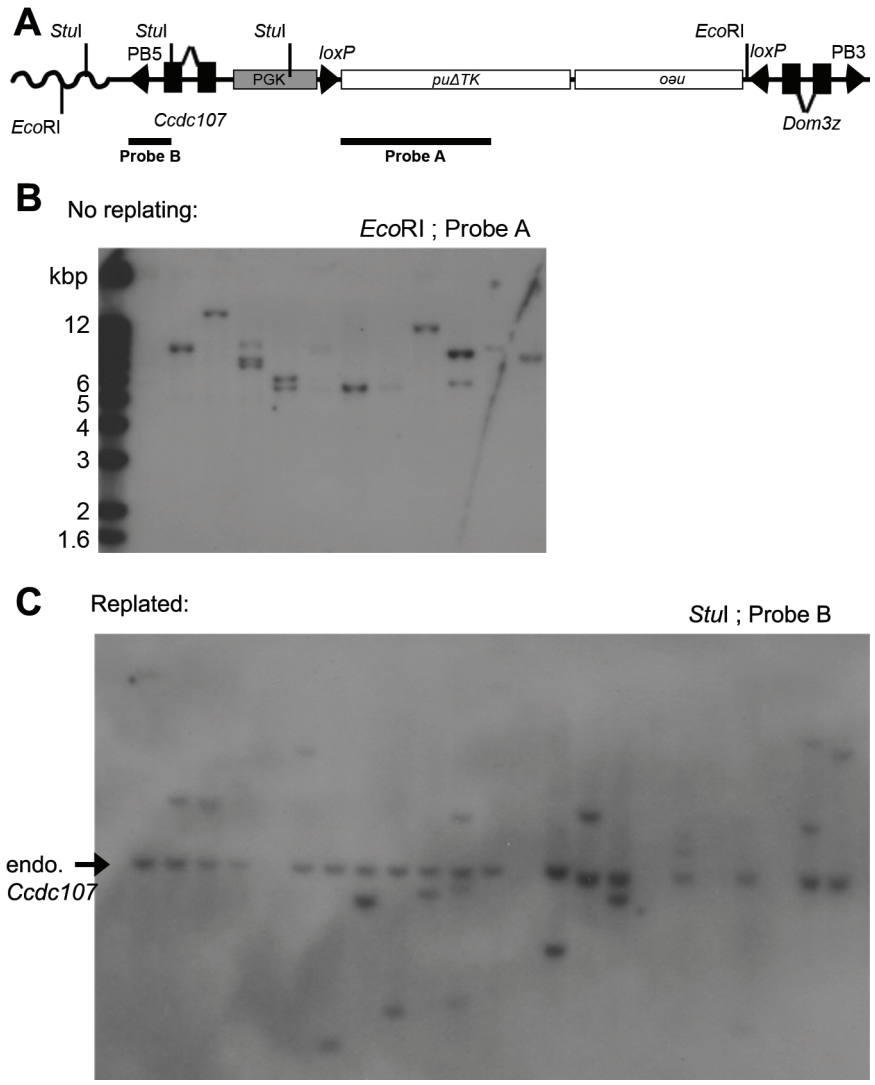


Figure 5.2: A—Map of transposon construct showing probes and restriction sites used. B—Clones picked without replating contain more than one insertion, but in different proportions. C—Replating after transfection resolves the multiple bands and reveals most clones to have a single insertion. Using probe B also detects a band corresponding to the endogenous copy of the *Ccdc107* gene.

Replating the cells after allowing time for the transposase activity to subside ensures that the colony picked is truly clonal (i.e. derived from a single cell with a stably-integrated transposon). I picked 96 clones from this second experiment to form the TNP100 arrayed library of heterozygous clones.

5.2.2 Mapping of insertion sites

I prepared DNA from a replica plate of this library and used splinkerette PCR (*Sau*3AI digest; see Methods) to amplify transposon-genome fragments. Sixty three PCR reactions gave a unique product (Figure 5.3A). I sequenced these fragments, and processed the sequences by clipping transposon sequence before the TTAA site and genomic sequence after any observed *Sau*3AI site. This removes chimaeric fragments that arise when two genomic fragments manage to ligate to each other before ligation to the splinkerette adaptors. I mapped the resulting fragments to the genome using SSAHA (Ning *et al.*, 2001). As both 5′ and 3′ fragments were amplified for each insertion, mapping confidence is highest when the fragments either side of the transposon map to the same locus, on opposite strands. In cases where only one side amplified a product, this can still be mapped. To ensure accurate mappings, I looked for a clearly visible transposon end and transition into genomic sequence before mapping these cases, and also required that the full length mapping was unique in the genome. Unambiguous mappings were obtained for 57 clones (Figure 5.3B). The insertion sites were spread across 17 chromosomes.

5.2.3 Generation of double resistant clones

I picked clones with successfully mapped insertions and expanded them to allow loss of heterozygosity to occur. I allowed the clones to expand to around five million cells on a 30 mm diameter (6-well) tissue culture plate, transfected a PGK-Cre expression plasmid by lipofection and transferred the cells to a 90 mm plate. This expansion is likely to be more than sufficient for LOH in most clones—based on the rate of LOH previously calculated (Luo *et al.*, 2000) an expansion to around 5,000 cells should be sufficient to observe one or more LOH events (Figure 5.4). However, as the transfection and locus-specific efficiency of Cre in this system will vary, I opted for a longer expansion period in these test experiments to increase the chance of observing and capturing homozygous mutants.

The day after plating I changed the medium to DBL medium (200 μg/ml G418 and 3 μg/ml puromycin). Some clones produced large numbers of double resistant cells, comparable to the number of cells plated. These clones are likely to have two copies of the construct, and were not analysed further. Some clones did not yield double resistant cells at all; in these cases the mutation could be homozygous lethal, or no LOH event occurred in the culture. However, some clones produced varying numbers of double resistant colonies, ranging from just a few to a few hundred (Figure 5.5).

The best way to characterise a population of clonogenic cells, such as the double resistant populations isolated here, is to pick and analyse subclones. I picked several colonies for each clone and genotyped them to investigate whether these cells represented real homozygous mutants.

5.2.4 Genotyping double-resistant clones

Southern blot to detect allelic transposon insertions

I designed a Southern blot probe to allow me to identify clones with two allelic copies of the transposon, and the relative amounts of TNP (*puro* oriented transposon) and TNN (*neo* orientation) contained in the cells (Figure 5.6A). The probe is a 1 kb *Sac*II–*Xmn*I restriction fragment of the transposon vector spanning the PB repeat and the *Ccdc107* exons. An *Nco*I site is present in this region that the probe will hybridise to, and also at the 5′ end of the *puro*Δ*TK* gene. Therefore, a different size *Nco*I fragment will be detected depending on the orientation of the resistance cassette: 1.7 kbp for TNN and 1.3 kbp for TNP. The other fragment detected by the probe is formed by the cut within the probe region and the closest *Nco*I site in the genome. The size of this fragment depends on the position of the insertion, and therefore allows discrimination between sites. Additionally, the probe detects two fragments of constant size from the endogenous *Ccdc107* gene—these can be used as a loading control.

I digested genomic DNA from double-resistant clones with *Nco*I, and probed the separated fragments with the probe described above. Two example clones are shown in Figure 5.6B. All subclones shown here contain four constant bands. Two of these are the predicted size for the endogenous *Ccdc107* bands, and the other two represent the TNN and TNP specific bands, as shown by hybridisation to digested plasmid. Homozygous mutants should have two copies of the transposon, one in each orientation, at a single locus. With the two endogenous bands, this should give five bands in total.

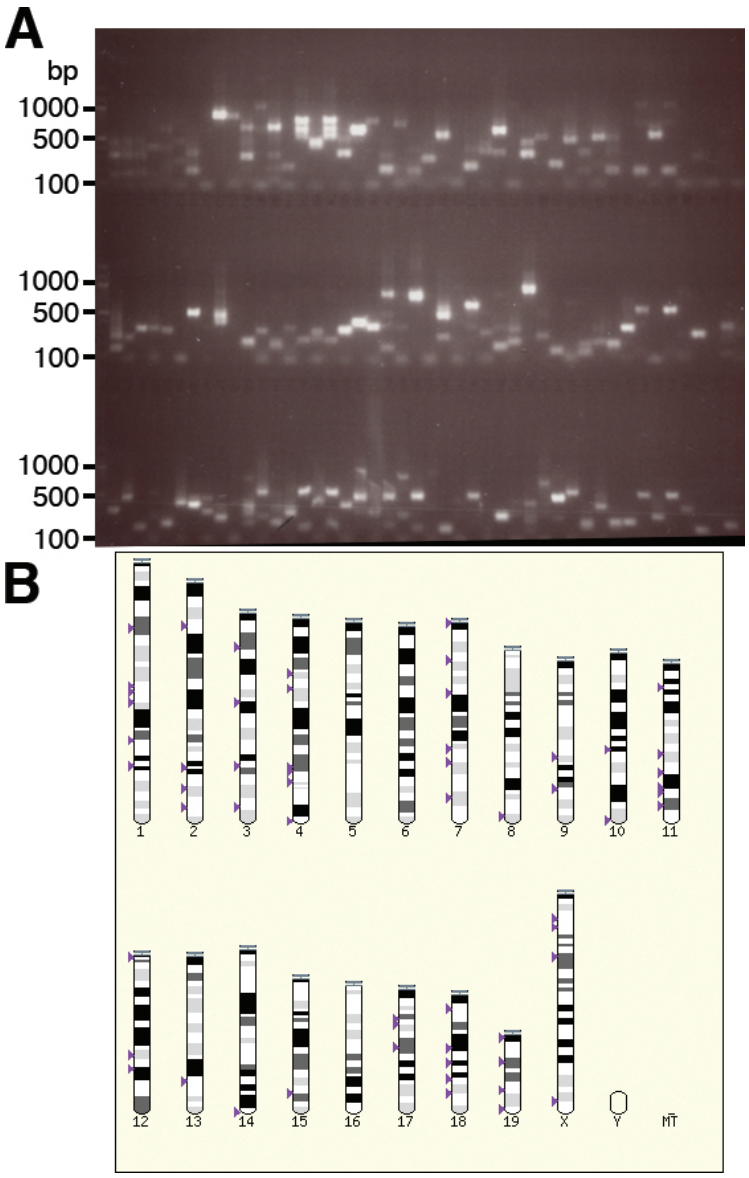


Figure 5.3: A—Second round splinkerette PCR products for the TNP100 set of clones. 5′ and 3′ products for each clone are loaded next to each other. B—Locations of successfully mapped PCR products

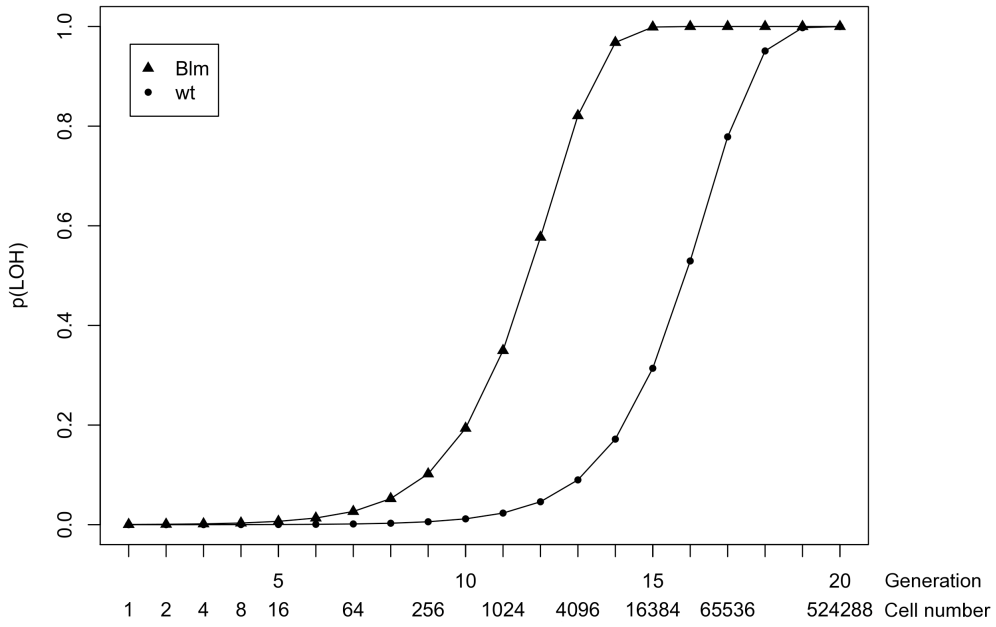


Figure 5.4: Predicted expansion time required to observe LOH events. The probability of at least one LOH event occurring at the specified generation is plotted: $1-(1-l)^n$ where n is the cell number at that generation and l is the LOH rate (rates for the *Gdf9* locus from Luo *et al.* 2000).

Clones with two copies of the transposon to begin with, at different loci, will have two locus-specific bands and therefore six in total. Both categories can be seen on the blot (Figure 5.6B). The clone with two non-allelic copies (F4) also contained two copies in the starting population as shown by Southern blot of clones from G418 selection only (Figure 5.6B lanes 1 and 2).

Selection background in initial experiments

For other clones from this experiment I observed a different result on the Southern blot. All subclones from one clone, and two out of six from another did not have both the *neo* and *puro* bands, despite surviving double selection (Figure 5.7A). These clones showed only the TNP band, indicating that they did not express *neo* from the PGK promoter, despite surviving G418 selection. However, during this experiment cells grew very slowly while under double selection. From other observations it emerged that this was due to the use of degraded L-glutamine in the lab culture media, rather than the double selection itself.

As G418 only kills actively dividing cells effectively, I considered whether slow growth when starved of L-glutamine, an essential amino acid, could explain the selection background, as I had not observed any background G418 resistance in previous experiments. By thawing replica plates of the double resistant subclones and reselecting in media containing fresh L-glutamine, I found that these cells were sensitive to G418 (and DBL) when grown in optimal culture conditions (Figure 5.7B). This highlights the importance of culture conditions in these selection experiments.

Thus, only double resistant subclones from one clone (G5) showed the expected band pattern in these experiments. I went on to analyse these in more detail.

5.2.5 Two classes of mutants are present in the double resistant population

Clones with two allelic copies of the transposon are potentially homozygous. To verify this, I checked to see whether the wild type locus was also present in these clones. I used a PCR assay with three primers in total—two locus-specific primers flanking the insertion site and one that hybridises to the PB transposon and extends into the genomic sequence. Homozygous mutants should only amplify the PB-

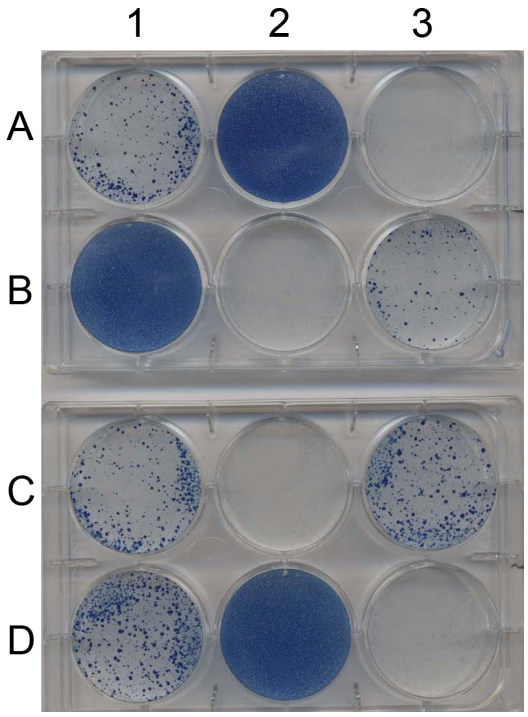


Figure 5.5: Typical results of double drug selection. Three classes of clone are visible: Those for which most cells plated are double resistant (e.g. row A, well 2), Clones that yield no double resistant cells (e.g. row A, well 3) and clones with varying numbers of double resistant colonies (e.g. row A, well 1; row B, well 3).

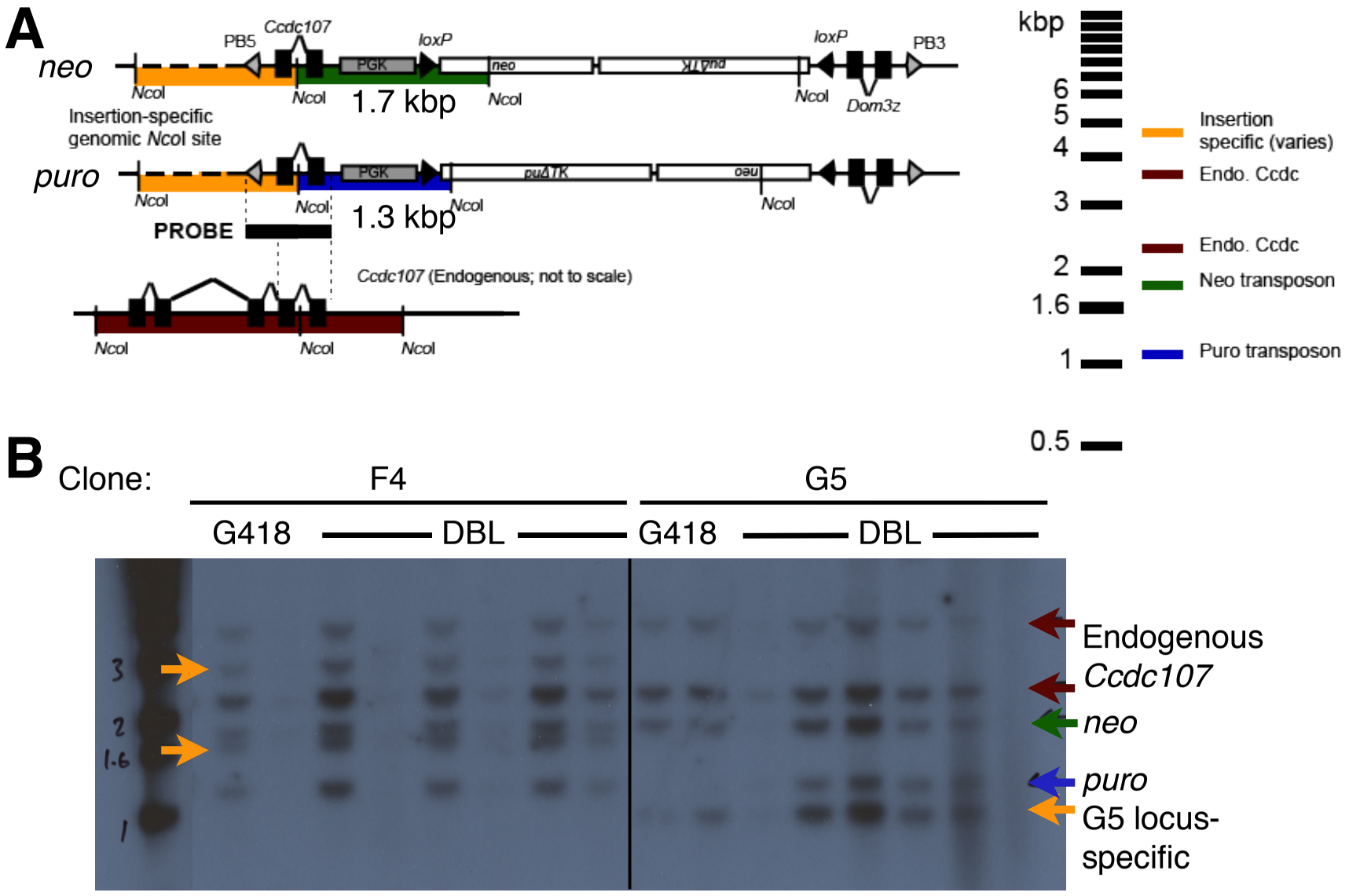


Figure 5.6: A—Probe used and predicted bands (see text) B—Examples of a clone containing two insertions at different loci (F4, left) and a potential homozygote with both *neo* and *puro* transposons at a single locus (G5, right).

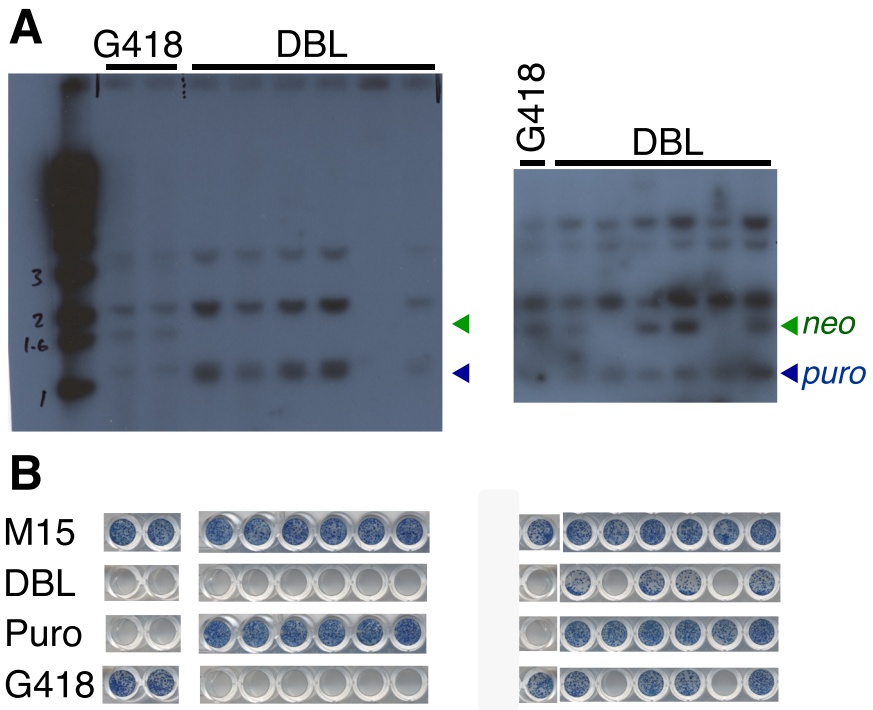


Figure 5.7: A—G418 and double-resistant (DBL) subclones from two separate clones, showing lack of TNN (*neo*) band. B—These clones show sensitivity to G418 and DBL when reselected.

genome junction product. A typical result is shown in Figure 5.8 for clone G5 with an insertion in the *Dymeclin* (*Dym*) gene. Three subclones do not amplify a wild type band, and are therefore homozygous. However, three subclones with an identical Southern blot pattern indicating two transposons at the *Dym* locus also amplified a clear wild type band. This suggests that more than two *Dym* alleles may be present in these cells—two mutant and at least one wild type.

I repeated the expansion and double selection procedure to obtain more double resistant cells for study and to ensure these results were not due to incomplete selection in the experiment above. Conditions for expansion and selection were the same, although I used electroporation to transfect the Cre plasmid. This time all double resistant clones had both *neo* and *puro* bands when analysed by Southern blot as above (Figure 5.9). This indicates that the selection worked effectively this time, when the cells grew at a normal rate. To simplify the process of isolating a larger set of double resistant clones for analysis, I also used a *Blm*-deficient cell line (NRB2) expressing a 4-hydroxytamoxifen (4-OHT) inducible Cre protein (see Chapter 2). This allows shorter expansion times to be used, as Cre induction is very efficient even in small cultures.

5.2.6 Summary of isolated double resistant clones

Altogether I isolated double resistant cells from 16 clones (Table 5.1). However, the results of PCR genotyping showed that some double resistant subclones still retained the wild type locus (Figure 5.10). The double resistant subclones generally comprised a mixture of genuine homozygous cells and cells that retain a wild type band in the PCR assay. Differences in expansion time, locus or Cre provision method did not appear to affect the general pattern, although these results do not allow this to be analysed systematically. The average clonal proportion of homozygotes obtained in all of these experiments was 34%, although as can be seen from the table, this can vary from 0–100%.

5.2.7 Double resistant clones retaining a wild type locus

It is possible that the wild type band in these PCR assays arises from a small proportion of wild type cells in the culture, either leftover feeder cells or cross-contamination from another mutant. To ad-

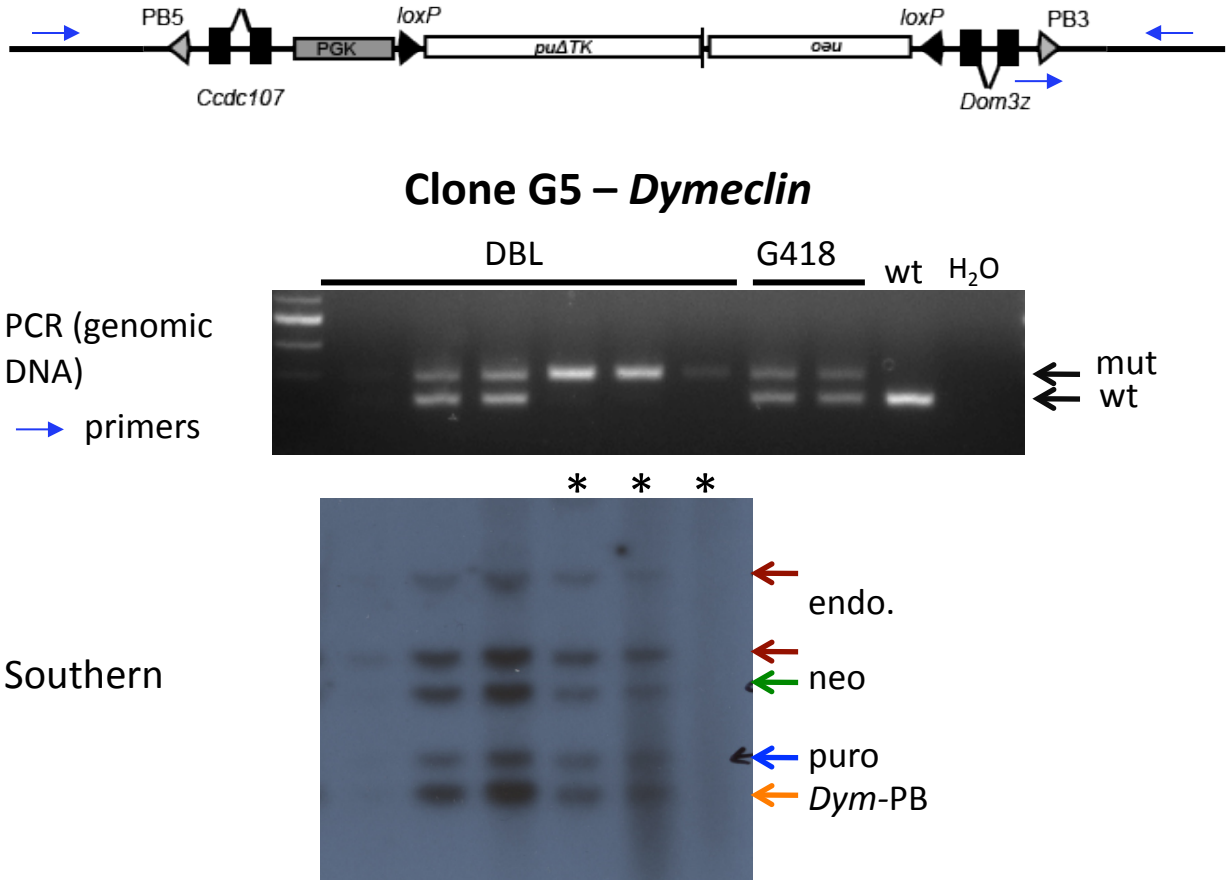


Figure 5.8: A—PCR using primers flanking insertion site and a transposon primer, indicated by arrows. In this case the mutant band is larger than the wild type band. Double resistant (DBL) clones 4–6(∗) only amplify the mutant band. B—Southern blot of the same subclones (reproduced from Figure 5.6).

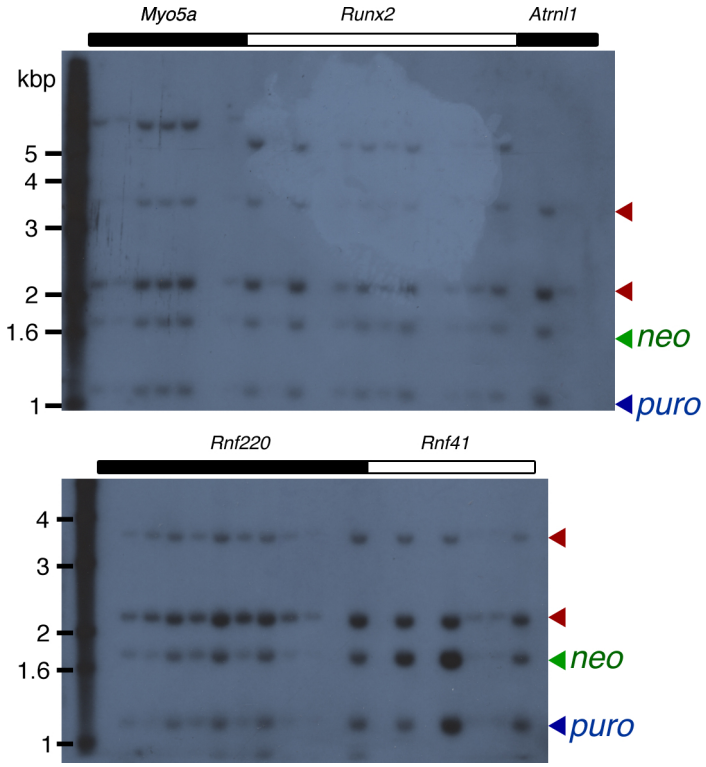


Figure 5.9: No selection background under normal selection conditions. Results for double resistant subclones from five clones with the indicated locus of insertion are shown, using the same Southern blot scheme as in Figure 5.6

Locus	Chr	Co-ordinates	Cre	Expansion	−/−	−/−/+$_n$	Fraction
Runx2	17	44,913,586	Electroporation	6w	10	0	100%
No gene (FL2.A11)	8	127,801,265	ERT2	96w	8	0	100%
Myo5a	9	74,990,083	Electroporation	6w	6	0	100%
Acpp	9	104,240,468	ERT2	96w	6	1	86%
No gene (FL2.C6)	9	15,258,570	ERT2	24w	4	3	57%
Dym	18	75,432,480	Lipofection	6w	3	3	50%
Arrb2	11	70,249,198	Electroporation	6w	2	4	33%
Rnf220	4	117,117,646	Electroporation	6w	2	6	25%
Sall1	8	91,577,321	ERT2	96w	2	5	29%
No gene (FL2.C1)	8	127,801,265	ERT2	24w	2	6	25%
Ddt	10	75,236,416	Lipofection	6w	1	3	25%
Itgb6	2	60,436,094	ERT2	24w	1	6	14%
Picalm	7	97,279,369	Electroporation	6w	0	1	0%
Atrn11	19	57,986,911	Electroporation	6w	0	1	0%
Macrod2/Flrt3	2	140,500,155	Electroporation	6w	0	0	N/A
Rnf41	10	127,863,548	Electroporation	6w	0	0	N/A
Srgap3	6	112,750,941	ERT2	96w	0	6	0%
Myh7b	2	155,429,901	ERT2	96w	0	6	0%
Tera	6	148,887,008	ERT2	96w	0	6	0%
Mrp133	5	31,916,879	ERT2	96w	0	6	0%
Tera	6	148,887,008	ERT2	96w	0	4	0%

Table 5.1: Results of genotyping for all double resistant clones

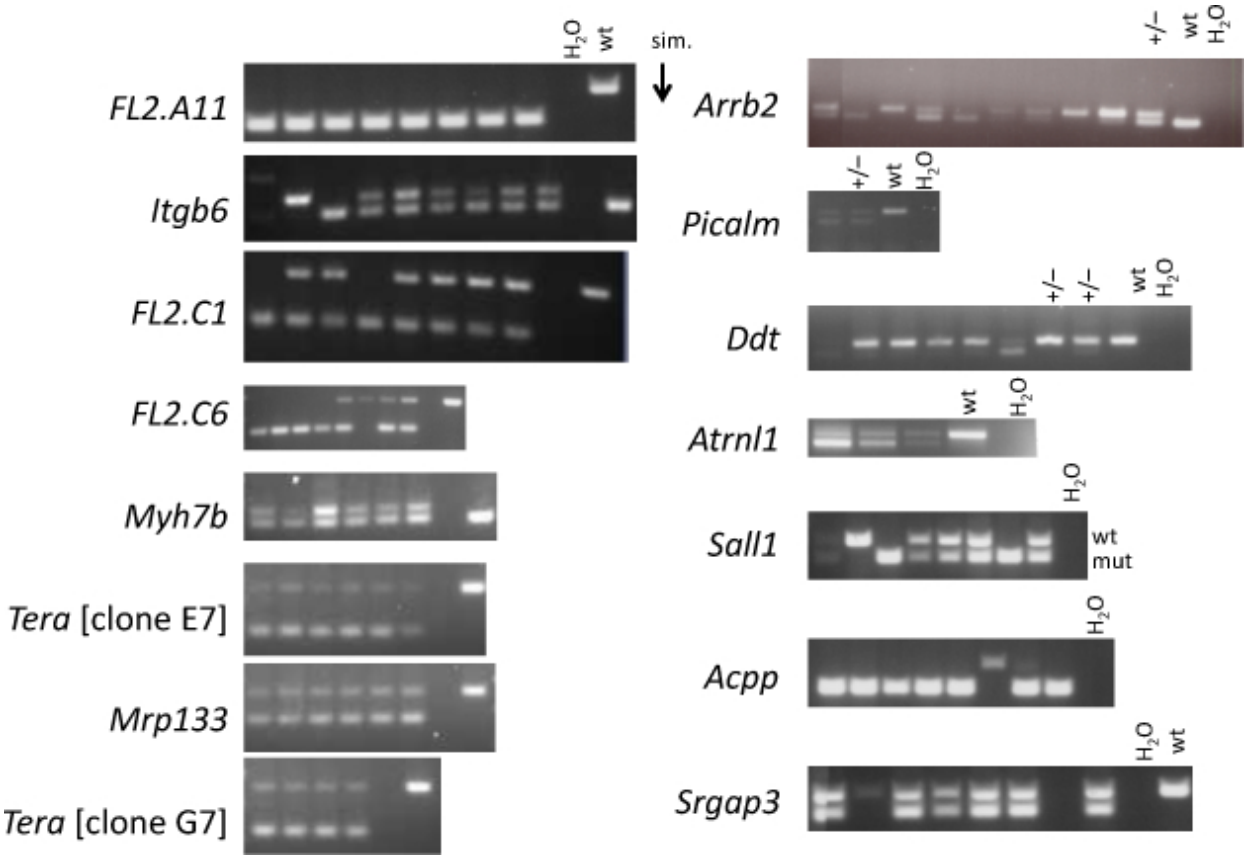


Figure 5.10: PCR genotyping of double resistant subclones from clones with an insertion at the indicated locus. Primers are designed to flank the insertion site, with an additional transposon primer as in Figure 5.8. H_2O, PCR without template; wt, PCR using wild type template DNA; +/–, PCR using DNA from cells heterozygous for the specific insertion.

dress this, I used Southern blotting with a probe specific to the individual insertion site, rather than the general transposon probe above. As the signal from a Southern blot is directly proportional to the amount of DNA this gives a more accurate representation of the relative amounts of mutant/wild type chromosomes in the culture. I analysed several clones in this way by stripping the original blot and reprobing with a probe designed to detect a different sized band for the wild type locus, the mutant with the *neo* transposon and the mutant with the *puro* transposon (Figure 5.11). For the *Myo5a* mutants, all subclones were homozygous as expected from the PCR result. In the case of the *Runx2* mutants, no wild type band was detected on the Southern blot, despite a clear band in the PCR assay. Therefore these are likely to be true homozygous mutants, and the wild type band is likely to result from contaminating cells below the level detectable by Southern blot.

Most interesting were the subclones from the *Rnf220* mutants for which three different classes can be seen on the blot (Figure 5.11B, right). Three bands were seen in wild type retaining clones (Figure 5.11B, (i)), corresponding to the *neo*, *puro* and wild type loci. The wild type band was approximately twice as intense as the others, indicating a ratio of 2:1:1 wild type:*neo*:*puro* chromosomes, and therefore possible tetraploidy. Two subclones homozygous by PCR assay were confirmed as such (Figure 5.11B, (iii)). These results show that two separate outcomes are possible after double selection, copy number increase with loss of wild type locus, presumably by *Blm*-related LOH, and copy number increase with retention of the wild type locus, which may be by acquisition of an abnormal karyotype.

These locus specific blots also highlighted the shortcomings of using PCR to assess homozygosity. The *Runx2* clones that gave a wild type band in the three-primer PCR were in fact homozygous when assessed by Southern blot. PCR is a much more sensitive technique than Southern blotting, so a small amount of contamination by wild type cells (which could be ES cells or cells from the feeder layer) may result in a wild type PCR product. Such low level contamination would not be detected on a Southern blot, where signal is directly proportional to the amount of DNA present. Therefore PCR genotyping alone may underestimate the real number of homozygous mutants, as in the case of the *Runx2* mutants in Figure 5.11 (middle).

Finally, some clones showed only a wild type band in PCR genotyping: for example *Myo5a* clone 6, *Runx2* clones 4 and 9 and *Rnf220* clones 4 and 5. In some cases (*Myo5a* and *Runx2*) no or very little DNA was isolated from these wells when I prepared DNA for Southern blots, so it is likely that these clones did not survive. When picking colonies I made a conscious effort to pick all kinds of morphologies, as to only pick "healthy looking" or large colonies may inadvertently select against genuine mutants. The wild type band in these cases where no ES cells grew may result from leftover feeder cells. However in the case of the *Rnf220* mutants, these "wild type only" subclones do show signal on the Southern blot, but do not in fact have an insertion at the *Rnf220* locus (see locus specific blot, Figure 5.11B(ii) and A. In part A the *Rnf220*-specific band is just visible at the bottom of the blot and appears to be absent in lanes 4 and 5). Therefore these may have arisen from mosaicism in the clone, despite the replating step.

As the result from the locus specific Southern blot indicated that the wild type retaining subclones may be tetraploid, I prepared metaphase spreads to check the karyotype of these subclones.

5.2.8 Karyotype of wild type retaining clones

Metaphase spreads prepared from wild type retaining subclones showed a clear near-tetraploid karyotype, whereas the genuine homozygotes isolated from the same clones (*Rnf220* and *Sall1*) had a normal diploid karyotype (Figure 5.12). Therefore in this case a change in ploidy had resulted in the transposon copy number increase that was then selected for. As both the diploid homozygotes and these tetraploid "wild type retainers" originated from a single cell with the PB insertion, this starting cell must have been euploid, and both LOH and ploidy changes must have occurred during the expansion phase.

5.2.9 DNA content analysis of wild type retaining subclones

As the wild type retaining double-resistant subclones examined above were tetraploid, I decided to explore whether these could be discriminated by DNA content analysis, as if this were possible then fluorescence activated cell sorting (FACS) could potentially be used to isolate the double resistant cells with a normal DNA content—i.e. homozygotes. Staining fixed nuclei with the DNA binding dye propidium iodide effectively discriminated the known near-tetraploid subclones from normal diploid cells (Figure 5.13A,B). However, running this analysis on a

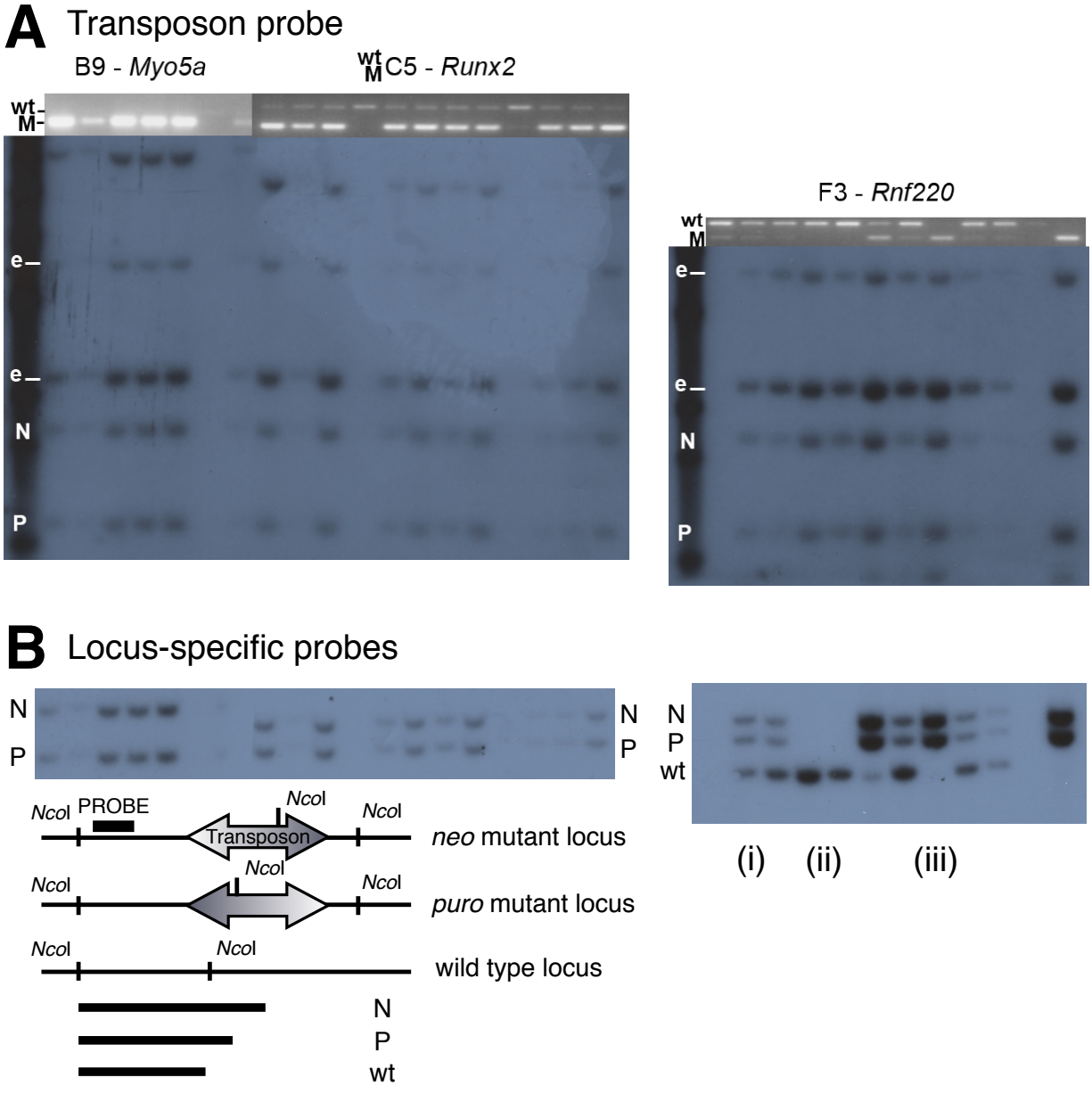


Figure 5.11: Double resistant cells analysed by Southern blot. A—Genomic PCR (original genotyping) and blot with transposon probe as in Figure 5.6. B—Reprobing of blot with a locus specific probe designed as shown. Two bands are seen for homozygotes, three for clones that genuinely retain the wild type locus. (i)–(iii): three genotype classes for *Rnf220* mutants; see text

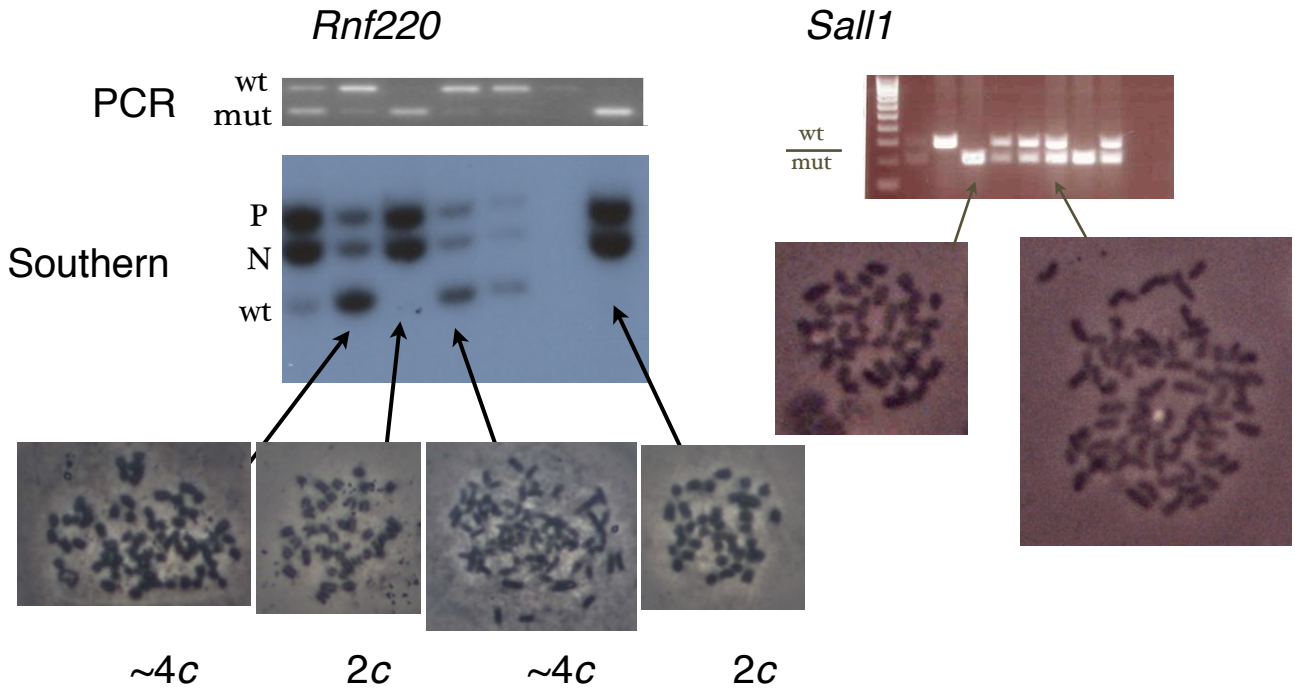


Figure 5.12: Representative chromosome spreads shown for the indicated clones. Spreads from clones that retain a wild type locus in the genotyping assays are tetraploid, whereas homozygous sister clones are euploid.

larger set of clones that had been determined to retain the wild type locus by PCR showed that most of these actually had a staining profile that resembled that of the known diploid subclones (Figure 5.13C).

It is possible that these clones had a less severe chromosome abnormality, such as a trisomy of the chromosome with the insertion or a segmental duplication. Indeed, a colleague's (Y. Huang, personal communication) double selection experiments isolated one such trisomic clone. Alternatively, the PCR assay used may be giving false negative results due to low level contamination and these clones may in fact be genuine homozygotes. The only way to be certain is to do the type of locus-specific Southern blot experiments above, which is labour intensive even on this small scale, and completely impossible on a genome wide scale.

5.2.10 The transposon disrupts transcription of genes when inserted into introns

The homozygous mutants isolated above gave me the opportunity to see if my transposon vector was mutagenic. I prepared RNA from double resistant subclones from three separate mutants with insertions in an intron—*Dym*, *Arrb2* and *Myo5a*. Using oligo-dT primers, I prepared cDNA by reverse transcription and used primers to exons flanking the intron with the insertion to see if a transcript was detectable. All clones that had been determined to be genuine homozygotes failed to amplify a PCR product (Figure 5.14). Therefore the transposon construct is mutagenic at the mRNA level. As this was the case in all three randomly picked insertion sites, the construct is likely to be mutagenic in most cases in which the insertion is in an intron.

5.3 Discussion

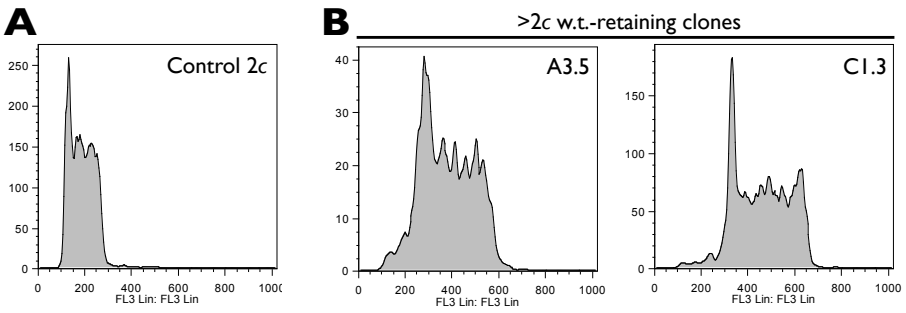
5.3.1 Paths to increase transposon copy number in *Blm* cells

Following the scheme above, I successfully isolated double resistant cells for many clones. Selection for cells with both *neo* and *puro* versions of the transposon was faithful, as on Southern blots I observed no background clones with only one version of the transposon. As expected, some clones initially contained two copies of the transposon due to the plasmid mobilisation system used. This would explain the clones that gave very large numbers of double resistant cells, although some clones which gave few enough colonies to pick also proved to have two insertions (Figure 5.6). This may reflect poor Cre efficiency at that particular locus, or poor Cre transfection efficiency for those clones.

Both genuine homozygous mutants and wild type retaining subclones were generally isolated from the double resistant population. The wild type retaining clones have increased the transposon copy by a non-LOH pathway, seemingly numerical chromosome instability (CIN). As both euploid and aneuploid cells were isolated from the same clone, which began as a single transfected cell, the original cell is likely to have been euploid. Therefore LOH and numerical CIN are competing pathways for transposon copy number increase in *Blm*-deficient ES cells. As both classes of double resistant subclones occur with similar frequencies, it could be inferred that the two processes have similar rates. However many of these experiments used relatively long expansions, and it is possible that tetraploid or trisomic cells may grow faster, as has been reported for some trisomies (Liu *et al.*, 1997). This would lead to increased representation in the selected population. Equally, some mutants may be at a fitness advantage or disadvantage, so the proportions of mutant and wild type retaining cells in the final population may not directly reflect the rate at which they arose.

5.3.2 Clones for which double resistant cells were not isolated

For 17 out of the 42 of the clones tested, no double resistant clones were isolated. This is unexpected, as even if the LOH rate at these loci is very low (e.g. if they are very close to the centromere), my results show that tetraploidy and trisomy are possible methods to acquire double resistance. Tetraploidy affects every chromosome. Therefore no location should be immune to copy number gain by this mechanism. Although it would have to be quite serious, a Cre position effect is a possibility. More likely is that LOH/other copy number gain is sufficiently rare for it not to occur in some cases, even though the expansion is quite prolonged in these cases. It is also possible that the gene is homozygous lethal when mutated, but even in this case it should still be possible to isolate aneuploid cells. For both cases where I had mapped the insertion (for the ERT-Cre experiments I only mapped the insertions after the double selection) but failed to isolated any double resistant cells, the insertions were on chromosomes for which I had previously isolated wild type retaining cells (*Macrod2* and *Rnf41*, Table 5.1). Thus, there does not appear to be a barrier to isolating



C

Clone	# wt-retaining subclones	2c	>2c	ND
A3	5	0	3	2
A4	1	1	0	0
A7	6	5	0	1
B4	6	5	0	1
C1	6	5	1	0

Figure 5.13: A—Wild type control DNA content profile. B—Examples of clones with near-tetraploid DNA content. C—Most wild type retaining clones have a near-2*c* DNA content.

cells with abnormal copy number of these chromosomes.

One further explanation could be a position effect with respect to expression of the resistance genes. Some loci may express sufficient levels of puro protein but not neo, due to their chromatin context or the influence of nearby regulatory elements. Although the two resistance genes are under the control of the same promoter and polyadenylation signals, the stability of mRNA and protein, and the amount required to confer resistance, is likely to differ.

5.3.3 Implications for creation of homozygous mutant libraries

In these experiments, where the expansion and double selection steps were done on a clone-by-clone basis, a mixed double resistant population was obtained in most cases. In most cases, the mixed population would not contain a sufficiently high proportion of homozygotes for genetic screens. Whether a proportion of 34% (the per clone average) would be sufficient to see a loss of function phenotype will depend on the assay used. For optimum performance, the double resistant population would have to be subcloned in order to create an arrayed library of pure mutant cells for genetic screens.

There is no way to select against cells with a wild type allele on a general basis. Therefore, to make a clonally pure library using the methods described here, double resistant subclones would need to be genotyped in order to identify the homozygous mutants. This also means that each insertion site would have to be mapped and a separate genotyping protocol designed. From a practical point of view, the effort required would be similar to serially targeting all known genes using the targeting vector and heterozygous ES cell resources that are quickly becoming available (International Mouse Knockout Consortium *et al.*, 2007). Ideally the library generation step would generate clones that could be picked and screened directly, and the insertion site only mapped once mutants of interest had been isolated.

An alternative strategy might be to reduce the expansion time to a critical level, such that only one LOH/CIN event is expected to occur (Figure 5.15). This would represent an expansion to a few thousand cells, roughly corresponding to a colony just visible to the naked eye. In this situation, a heterozygous clone would only rarely give rise to the mixed double resistant population and instead produce a double resistant population composed of either all homozygotes or all wild type retaining cells

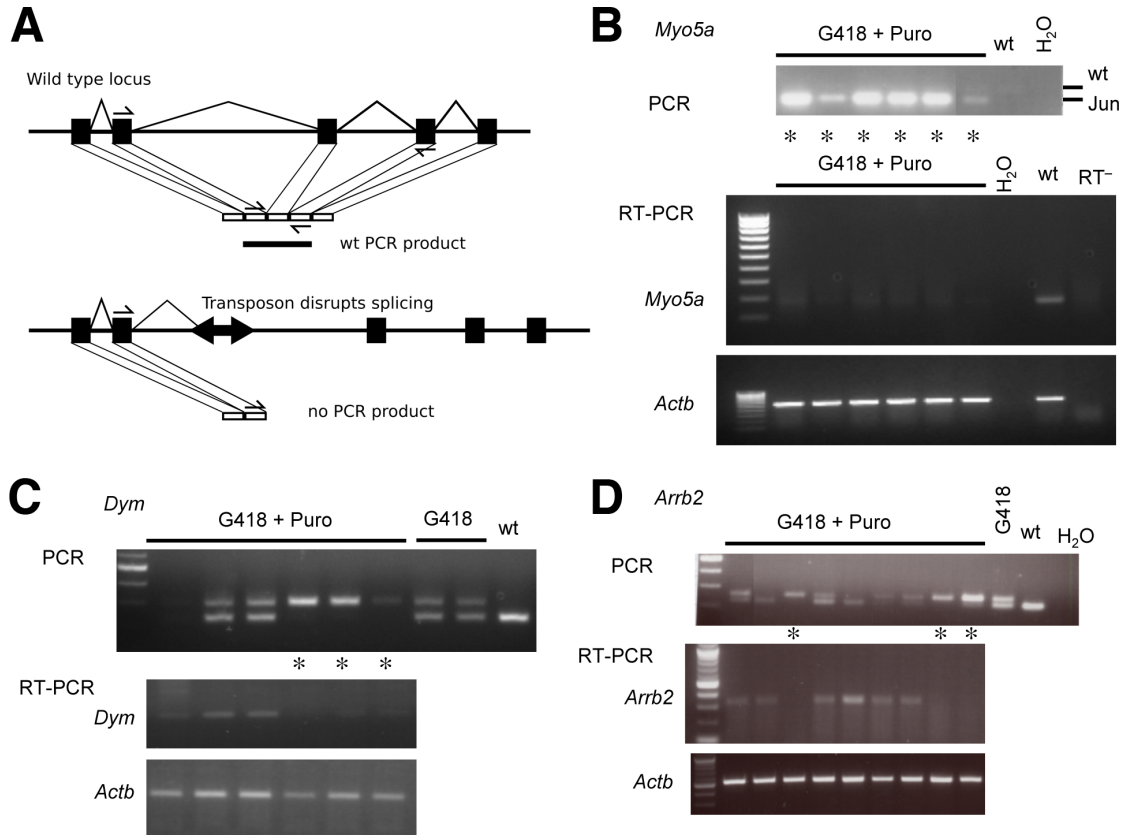


Figure 5.14: RT-PCR analysis of cDNA prepared from double resistant subclones. The corresponding genotyping PCR on genomic DNA is shown at the top of each sub-figure. RT-PCR reaction shown below, with *Actb* positive control at the bottom. A—*Dym*. B—*Myo5a*. C—*Arrb2*.

(Figure 5.15B,C). In this situation, the double resistant cells would be a pure population that could be screened directly. Retaining cells would become "passengers" in the library, so it is essential that the frequency of homozygotes obtained at this limit is high enough to give a complex and useful library.

However, using this limited expansion, the probability of isolating any double resistant cells at all from a given clone would also drop. For this reason it may be better to carry out the expansion and double selection in parallel in a pooled format, to avoid expanding many individual clones that do not yield double resistant cells. This would require the copy number of the transposon to be strictly limited to one at the start, so that there would be no clones with two copies from the beginning of the expansion, which would dominate over the low number of homozygous cells. Targeting the transposon to the X chromosome and mobilising from there would be one way to do this.

5.3.4 Conclusions

In this chapter I have demonstrated that the TNN-TNP inverter construct can be used to isolate homozygous mutants from expanded populations of *Blm*-deficient ES cells. Additionally, the construct disrupts transcription when inserted into introns and is thus likely to be an effective mutagen. An alternative pathway to increase transposon copy number exists via numerical chromosomal instability, thus the double resistant population is not purely homozygous mutants. The average clonal proportion of homozygotes was 34%, representing a significant enrichment for homozygous mutants. The clone-by-clone method for homozygote enrichment described in this chapter requires two subcloning steps to obtain pure homozygous populations, which is not practical on a genome wide scale. The next steps, described in Chapter 6 were to make a suitable transposon donor locus on the X chromosome to limit the initial copy number for library generation on a large scale.

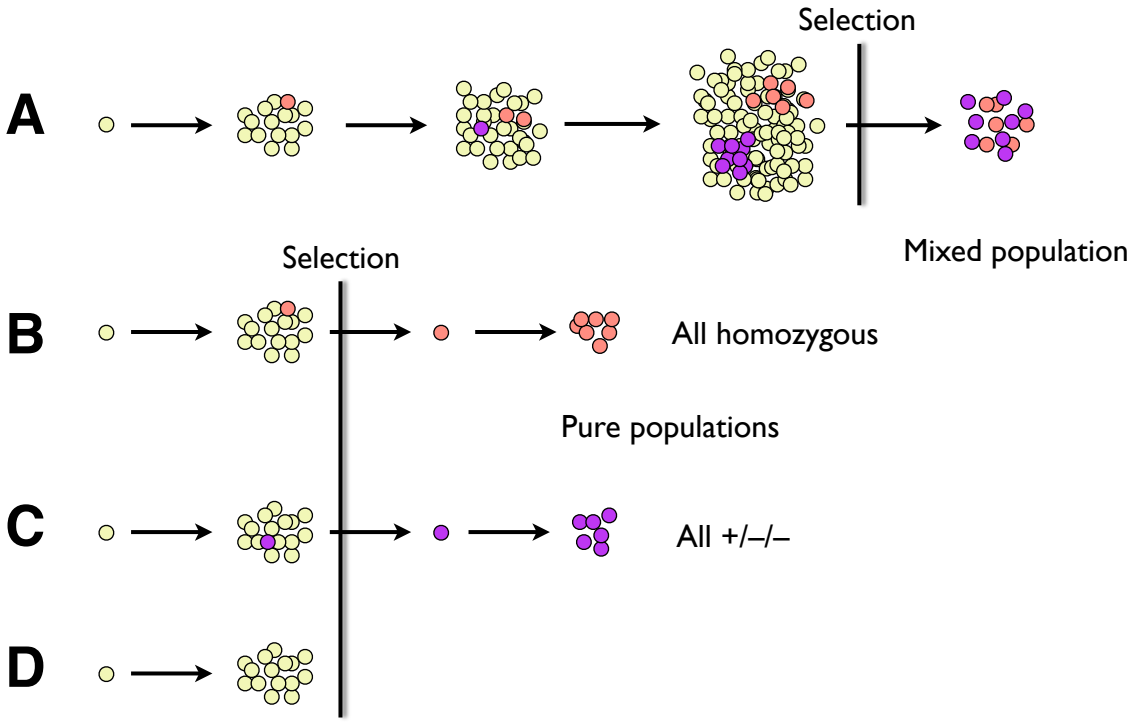


Figure 5.15: Possible consequences of over-expanding clones prior to double selection. A—Long expansion times allow two or more events to occur, resulting in a mixed double-resistant population. B, C—Ideal situation where only one event occurs, resulting in pure clonal double resistant populations. D—However, in many cases no LOH or copy number gain will occur.