CHAPTER THREE:

A *Brca1* CONDITIONAL

ES CELL SYSTEM

3.1 INTRODUCTION

3.1.1 *Brca1* conditional cell system – an overview

Several knockout alleles of mouse *Brca1* had been published when this project commenced (Table 1.3). Mice carrying these alleles did not accurately model human phenotypes: in direct contrast to human mutation carriers, murine heterozygotes had no increased predisposition to tumourigenesis. Furthermore, the embryonic lethality of homozygous knockout mice precluded extensive studies of the molecular effects of the loss of *Brca1*. However, murine embryonic stem (ES) or mouse embryonic fibroblast (MEF) cell lines carrying *Brca1* knockout alleles have been used in functional studies, especially of the response of mutant cells to DNA damage. These studies demonstrated that murine Brca1, like its human homologue, appears to have caretaker roles in the cell.

To extend the understanding of the functions of Brca1, the first aim of this project was to generate a conditional *Brca1* ES cell line (consisting of one null allele and one conditional allele of the gene (Figure 1.16)) for use in a genome-wide screen for suppressors of *Brca1*.

3.1.2 Conditional ES cells in a suppressor screen/preclusion of the screen

Conditional *Brca1* ES cells were to be used in a screen for suppressors of the lethality of *Brca1*. Cells would be subjected to genome-wide mutagenesis through retroviral-mediated gene trapping before Cre-mediated deletion of the conditional allele (Figure 1.18). Cells carrying a trapped and mutated suppressor gene were expected to be viable following loss of the second allele of *Brca1*. Gene trapping on a genome-wide scale has been successfully performed by colleagues Ge Guo and You-Tzung Chen, and large scale gene trapping efforts are underway at several institutions, demonstrating that this is a viable form of mutagenesis for genome-wide screens (Hansen, 2003; Stryke, 2003). Retroviral-mediated delivery of gene

trap cassettes into ES cells is very efficient (Soriano, 1991). As loss of *Brca1* in ES cells is expected to be a lethal event, and selection markers are carried by both *Brca1* alleles and by the gene trap cassette, the screening criteria are highly stringent. Both the knockout and conditional knockout alleles target exon 2 of *Brca1*, both because it contains the translational start site, and because a previous *Brca1* exon 2 knockout allele behaves as a null allele (Ludwig, 1997).

The existence of suppressors of *Brca1* is supported mainly by studies of another *Brca1* deletion allele: mice homozygous for an allele of *Brca1* coding for a truncated Brca1 protein were fully viable on certain genetic backgrounds, but not on others (Ludwig, 2001). There is additionally a published report of a woman homozygous for a cancer-related *BRCA1* mutation (Boyd, 1995), but this finding has been disputed and attributed to a PCR error (Kuschel, 2001).

As it is possible that suppressors of *Brca1* might be either dominant or recessive, a modification was made to the screen to allow identification of recessive suppressors as well as dominant ones. It has been shown previously that in ES cells lacking the Bloom's Syndrome RecQ helicase homologue (*Blm*), mitotic recombination and loss of heterozygosity (LOH) occur at an increased rate (approximately 20-fold higher than that of wildtype cells (Luo, 2000)). Therefore, *Blm*-deficient ES cells carrying a gene trap at a given locus may undergo LOH at the trapped locus. Half of such events would result in homozygosity of the gene trap at the locus, allowing recessive suppressors to be trapped. This technique has been successfully used by Ge Guo, a colleague, to identify recessive genes involved in the mismatch repair pathway.

However, one prerequisite for successful screening was that the excised conditional allele was a null allele. This was not the case; the conditional allele generated in this study did not behave like previously-described null alleles after excision. This precluded the use of the *Brca1* conditional ES cells in a gene trap suppressor screen. The viability of the excised conditional allele was unexpected, but meant that cells carrying this allele could be used

for functional studies. Additionally, mice carrying some of the alleles discussed in this chapter have been generated. These mouse models will be discussed further in Chapter 4.

3.2 RESULTS

3.2.1 *Brca1*$^{-}$ behaves like previously-described null alleles of *Brca1*

The hallmarks of previously-described null knockout alleles of *Brca1* include an inability to generate double-targeted ES cells, embryonic lethality of homozygous mutant mice, and no increased predisposition to cancer in heterozygous mutant mice (see Table 1.3). In this study, exon 2 of *Brca1* was first targeted with a replacement targeting vector (*Brca1*-Hprt-TV) containing a *Hypoxanthine phosphoribosyltransferase* (*Hprt*) minigene transcribed in the opposite direction from transcription of the *Brca1* allele, as well as a *thymidine kinase* (*tk*) gene from herpes simplex virus type I (HSV), used for negative selection against random integration (Figure 3.1a and Table 2.1). The resulting targeted allele is designated *Brca1*^{Brdm1}, and is referred to in this work as "*Brca1*$^{-}$" or simply "–" when in conjunction with another allele. *Brca1*-Hprt-TV is derived from *Brca1*-Neo-TV, a replacement targeting vector used by Ludwig *et al*, which replaces exon 2 with a *Neo* cassette. The allele generated using *Brca1*-Neo-TV behaved as a null allele (Table 1.3 #1) (Ludwig, 1997).

Brca1-Hprt-TV successfully targeted the *Brca1* locus at a targeting frequency of 12.5% (Figure 3.1b and c). However, an attempt to target the second, wildtype *Brca1* allele of *+/–* ES cells using *Brca1*-Neo-TV from Ludwig *et al.* was unsuccessful, demonstrating that double-targeted ES cells are either rare or cannot be generated, similar to what has been observed for previously-described null alleles of *Brca1* (Hakem, 1997; Ludwig, 1997).

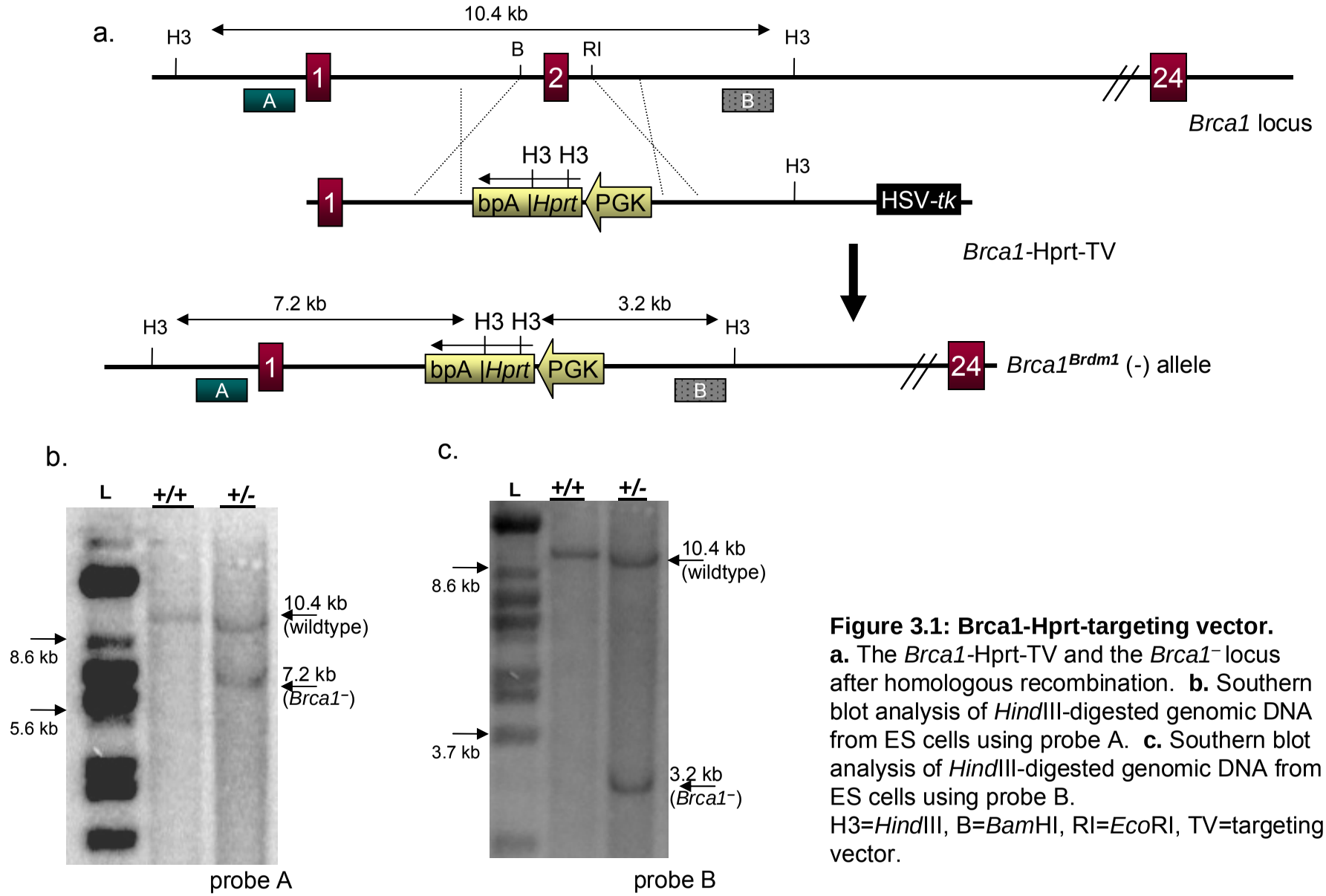


Figure 3.1: Brca1-Hprt-targeting vector. **a.** The *Brca1*-Hprt-TV and the *Brca1*$^{-}$ locus after homologous recombination. **b.** Southern blot analysis of *Hind*III-digested genomic DNA from ES cells using probe A. **c.** Southern blot analysis of *Hind*III-digested genomic DNA from ES cells using probe B.
H3=*Hind*III, B=*Bam*HI, RI=*Eco*RI, TV=targeting vector.

3.2.2 Conditional alleles of *Brca1*

3.2.2.1 Generating a conditional allele of Brca1

The construction of the conditional allele was more complex than initially expected. To reduce confusion and briefly summarize: initial cloning of the *Brca1* conditional targeting vector *Brca1*-cond1-TV included two cloning mistakes, which were discovered serially. Two additional targeting vectors (*Brca1*-fixPuro-TV and *Brca1*-addPGK-TV) were constructed to fix the cloning errors. A final version of the conditional vector (*Brca1*-cond2-TV) was also generated. Figure 3.2 shows a schematic of the targeting events described in this chapter, and Figure 3.3 gives an overview of the alleles discussed in this chapter. Additionally, Table 2.1 describes the targeting vectors, the alleles they generate, and genotyping digests and probes.

3.2.2.2 Targeting the c1 conditional allele

The *Brca1* conditional targeting vector *Brca1*-cond1-TV was designed to flank exon 2 of *Brca1* with *loxP* sites and a split *Puromycin* (*Puro*) cassette (Figure 3.4a). The purpose of the split resistance cassette was to allow selection of ES cells carrying the excised conditional allele in culture, a requirement for the suppressor screen. The targeting vector also carried a *loxP*-flanked *Neo* cassette for selection of targeted events, and an HSV-*tk* gene for negative selection against random integration of the vector. *Brca1*-cond1-TV was electroporated into both *+/–* and wildtype ES cells, and targeted at ~10% efficiency (Figure 3.4b and c). The allele generated using *Brca1*-cond1-TV is designated $Brca1^{Brdc1}$. In this work, this allele is referred to as "*c1*," or, when the *Neo* selection cassette is retained, "*c1(+Neo)*".

3.2.2.2.1 Cre excision of +/c1 cells reveals a cloning mistake: Before using *–/c1* ES cells in the suppressor screen, *+/c1* cells were subjected to Cre electroporation and selection in puro-containing medium to assess the functionality of the reconstituted *Puro* cassette in culture. No puro-resistant colonies resulted from an electroporation of 10^7 *+/c1* cells. The Cre used in the electroporation was in general use in the lab at the time and normally

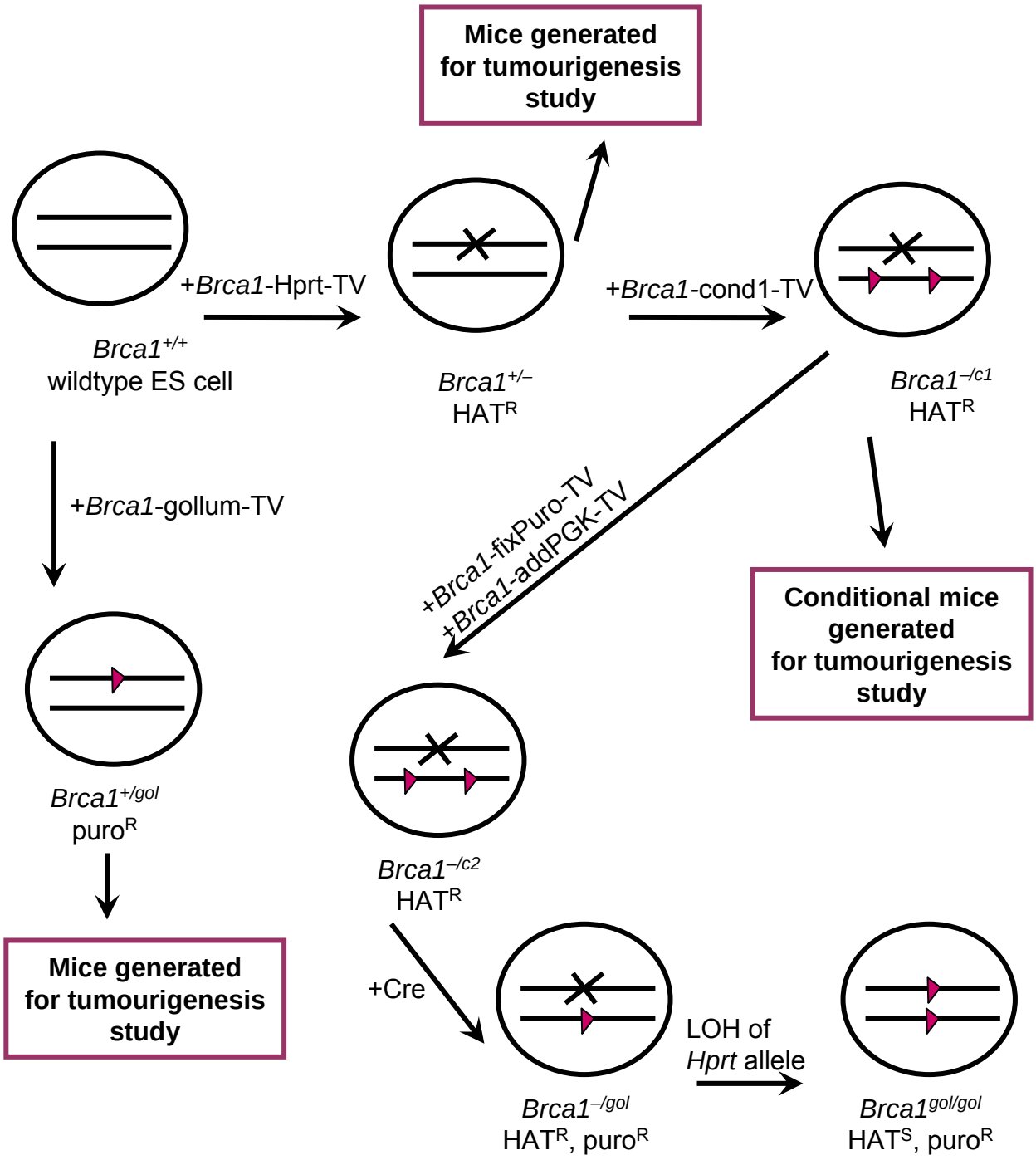


Figure 3.2: Overview of targeting events and generation of ES cells for blastocyst injection. This schematic shows the various targeting events described in this work. First, *Brca1*-Hprt-TV was electroporated into wildtype ES cells. These cells were used to generate mice and also for targeting of the *c1* conditional allele, then used to generate *Brca1* conditional mice. Correction of the *c1* allele gave the *c2* conditional allele. Cre-mediated excision of *c2* gave the viable allele *gollum* (*gol*). To generate *gol/gol* ES cells, LOH at the *Brca1* locus was selected for using 6TG selection against the *Hprt* gene carried by the *Brca1*$^{-}$ allele. *Brca1*-gollum-TV was used to generate *+/gol* ES cells for generating mice carrying the *gol* allele. R=resistant, S=sensitive.

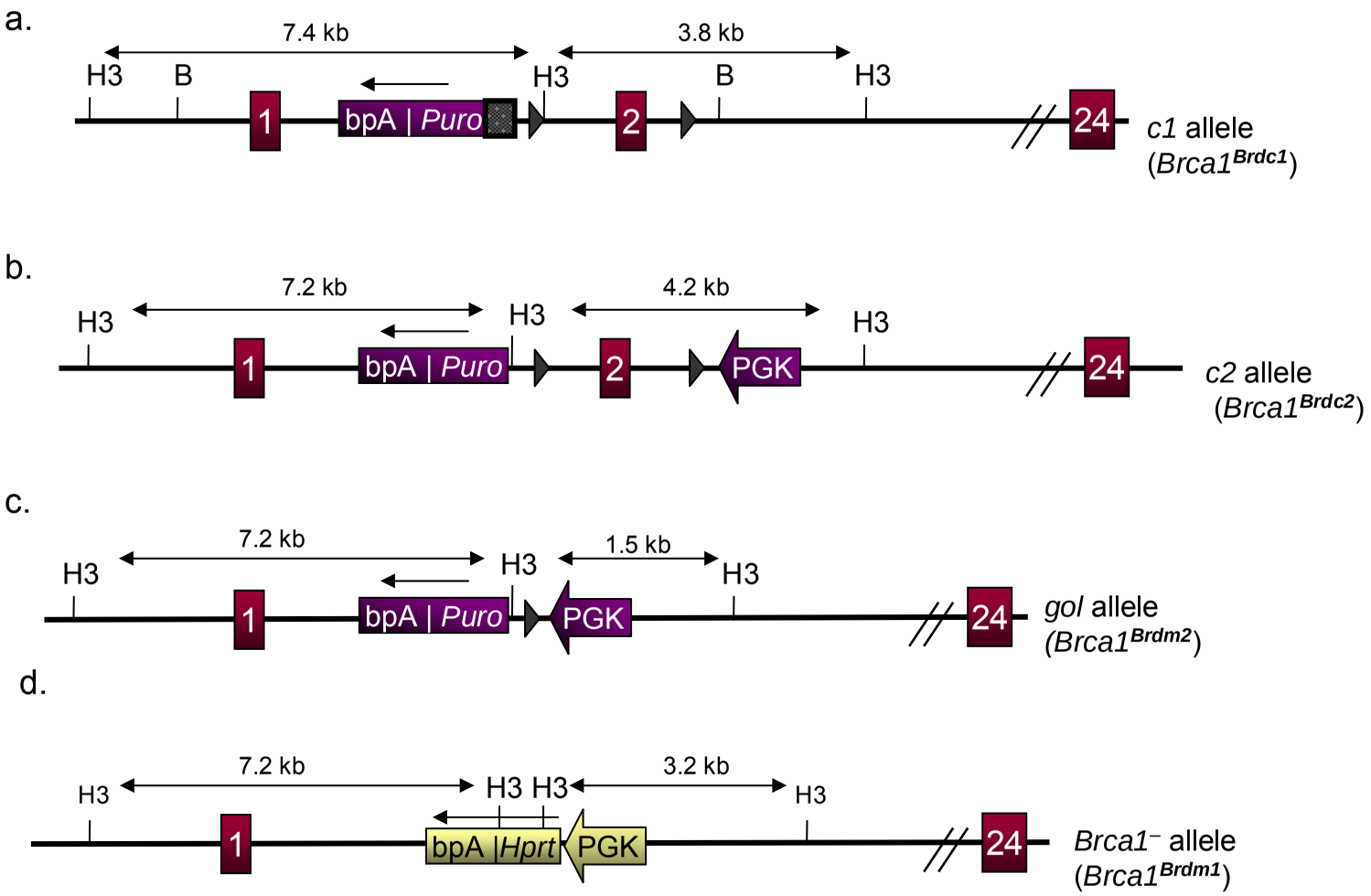


Figure 3.3: Overview: the alleles generated in this study.

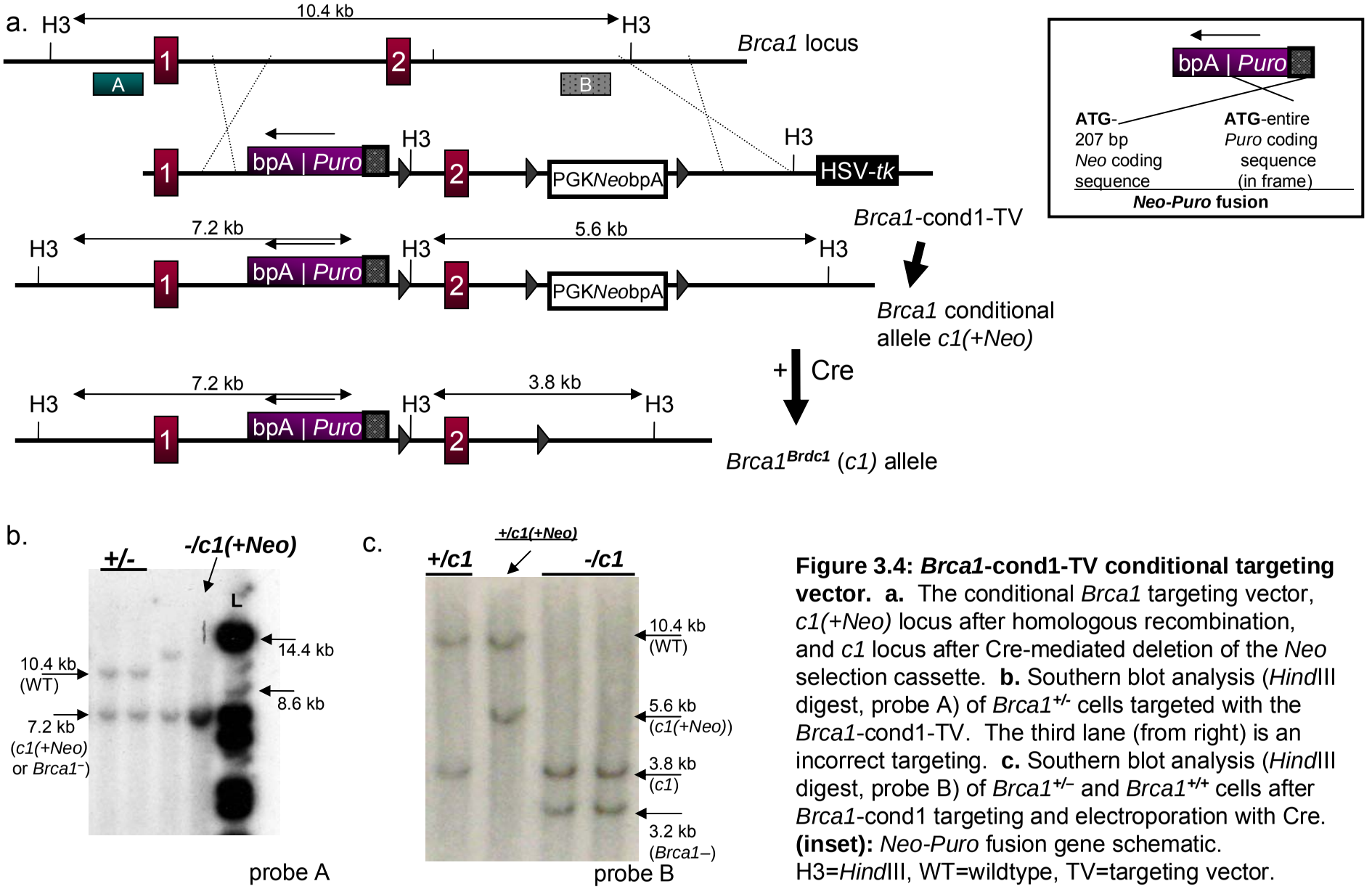


Figure 3.4: *Brca1*-cond1-TV conditional targeting vector. a. The conditional *Brca1* targeting vector, *c1(+Neo)* locus after homologous recombination, and *c1* locus after Cre-mediated deletion of the *Neo* selection cassette. **b.** Southern blot analysis (*Hind*III digest, probe A) of $Brca1^{+/-}$ cells targeted with the *Brca1*-cond1-TV. The third lane (from right) is an incorrect targeting. **c.** Southern blot analysis (*Hind*III digest, probe B) of $Brca1^{+/-}$ and $Brca1^{+/+}$ cells after *Brca1*-cond1 targeting and electroporation with Cre. **(inset):** *Neo-Puro* fusion gene schematic. H3=*Hind*III, WT=wildtype, TV=targeting vector.

resulted in an approximately 10% excision efficiency, so this lack of colonies was unexpected.

Sequencing of the *Puro* coding region of *Brca1*-cond1-TV revealed that, during cloning of the *loxP* site next to the *Puro* coding sequence, a ~200 bp section of the *Neo* coding region had also been included (see inset, Figure 3.4). This *Neo* insertion was in-frame with the *Puro* sequence, and contained an in-frame AUG start codon, which suggested that a Neo-Puro fusion protein might be generated. However, the lack of colonies resulting after puro selection of Cre-excised *+/c1* cells suggested that this fusion was not fully functional.

In hopes of finding a concentration of puro which maximized recovery of true puro-resistant colonies with a minimum of background colonies carrying the un-excised *c1* allele, *+/c1* cells were re-electroporated with Cre and selected at lower doses of puro (3.5 – 1.5 μg/ml vs. the original 5 μg/ml). No colonies were observed on plates selected in 3 or 3.5 μg/ml puro. While colonies did grow on a plate selected in 2.5 μg/ml puro, only the pool of colonies resulting after 1.5 or 2 μg/ml puro selection yielded enough genomic DNA for Southern blot analysis. In both these cases, a band corresponding to the un-excised *c1* allele was clearly visible (Figure 3.5). Therefore, as stringent selection of the excised form of *c1* was not possible, the *c1* allele could not be used for the suppressor screen.

The *c1* allele was still acceptable for generation of a conditional *Brca1* mouse model, as the *loxP* sites of *c1* are fully functional and exon 2 is efficiently excised following Cre expression. Mice carrying the *c1* conditional allele will be discussed further in Chapter 4.

3.2.2.3 Correction of the Neo-Puro cassette does not restore functionality of the conditional allele

A targeting vector (*Brca1*-fixPuro-TV) was constructed to replace the mutated *Puro* coding sequence with the correct sequence (Figure 3.6a). This vector targeted a corrected version of the *Puro* coding region into the *c1* allele. It

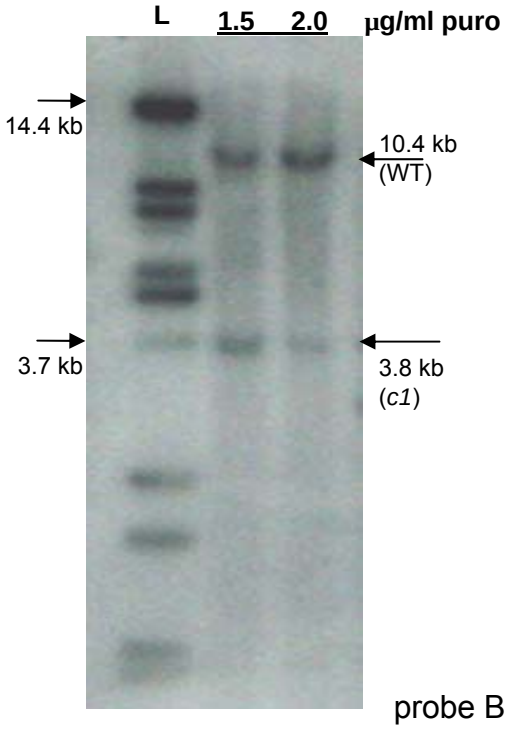


Figure 3.5: The *c1* allele is not efficiently excised by low doses of puro. *+/c1* ES cell colonies selected in 1.5 or 2 μg/ml puro were analyzed by Southern blot (*Hind*III digest, probe B). The *c1* allele (3.8 kb) is slightly less prevalent in cells selected at 2.0 μg/ml, but the background of un-excised *c1* allele is very high. L=ladder, WT=wildtype.

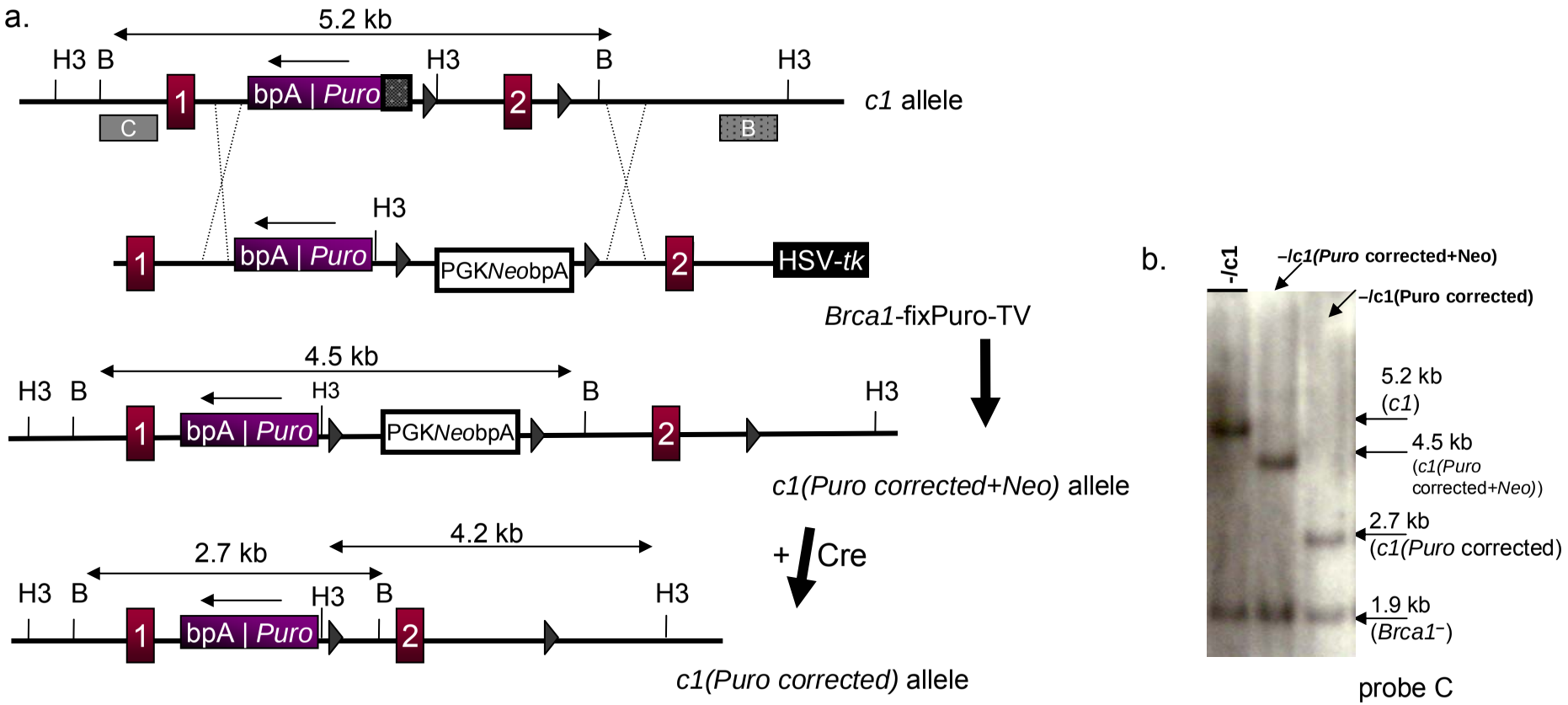


Figure 3.6: *Brca1*-fixPuro targeting vector. a. The *c1* allele and the *Brca1*-fixPuro-TV. After integration and Cre-mediated excision, the mutated *Neo-Puro* gene is replaced. **b.** Southern blot analysis (*Bam*HI digest, probe C) of *–/c1* cells after targeting and Cre-mediated excision of the selection cassette.
B=*Bam*HI, H3=*Hind*III, TV=targeting vector.

also carried a *loxP*-flanked copy of *Neo* for selection of correctly-targeted cells in culture. *Brca1*-fixPuro-TV was electroporated into *+/c1* and *–/c1* ES cells. To save time, G418-resistant colonies were pooled and re-electroporated with Cre before being screened by mini-Southern. The allele generated from correction of the *c1* allele by this vector is referred to as "*c1(Puro corrected)*" or, when the selection cassette is retained, "*c1(Puro corrected+Neo).*" Both *+/c1*(*Puro corrected*) and *–*/*c1*(*Puro corrected*) ES cell lines were generated (Figure 3.6b).

However, when *+/c1(Puro corrected)* ES cells were electroporated with Cre, no puro-resistant colonies were observed. Sequencing indicated that while the *Puro* coding region was correct, the PGK promoter intended to drive expression of the split *Puro* cassette had been lost during a cloning step of *Brca1*-cond1-TV. The missing promoter explained why the *Neo-Puro* fusion gene described above was not functional in *+/c1* ES cells following Cre-mediated excision of the *c1* allele.

As the excised version of the *c1(Puro corrected)* allele could not be selected in culture, this allele could not be used for the planned suppressor screen in ES cells. However, the lack of a PGK promoter does not effect the validity of the *c1* mouse model, as the *loxP* sites of *c1* are still fully functional and exon 2 is efficiently excised following Cre expression.

3.2.2.4 The Brca1-addPGK-targeting vector repairs the Puro cassette

Another targeting vector (*Brca1*-addPGK-TV) was designed to insert the PGK promoter of the *Puro* cassette into the *c1(Puro corrected)* allele. This vector carries a PGK promoter and a *loxP*-flanked *Neo* cassette for selection of targeted cells in culture (Figure 3.7a). *Brca1*-addPGK-TV was electroporated into *+/c1(Puro corrected)* and *–/c1(Puro corrected)* cells. The allele resulting from this electroporation is designated $Brca1^{Brdc2}$. In this work, this allele is referred to as "*c2*," or, when the *Neo* selection cassette is retained, "*c2(+Neo)*." *–/c1(+Neo)* ES cell lines were recovered, and were electroporated with Cre to remove the *Neo*

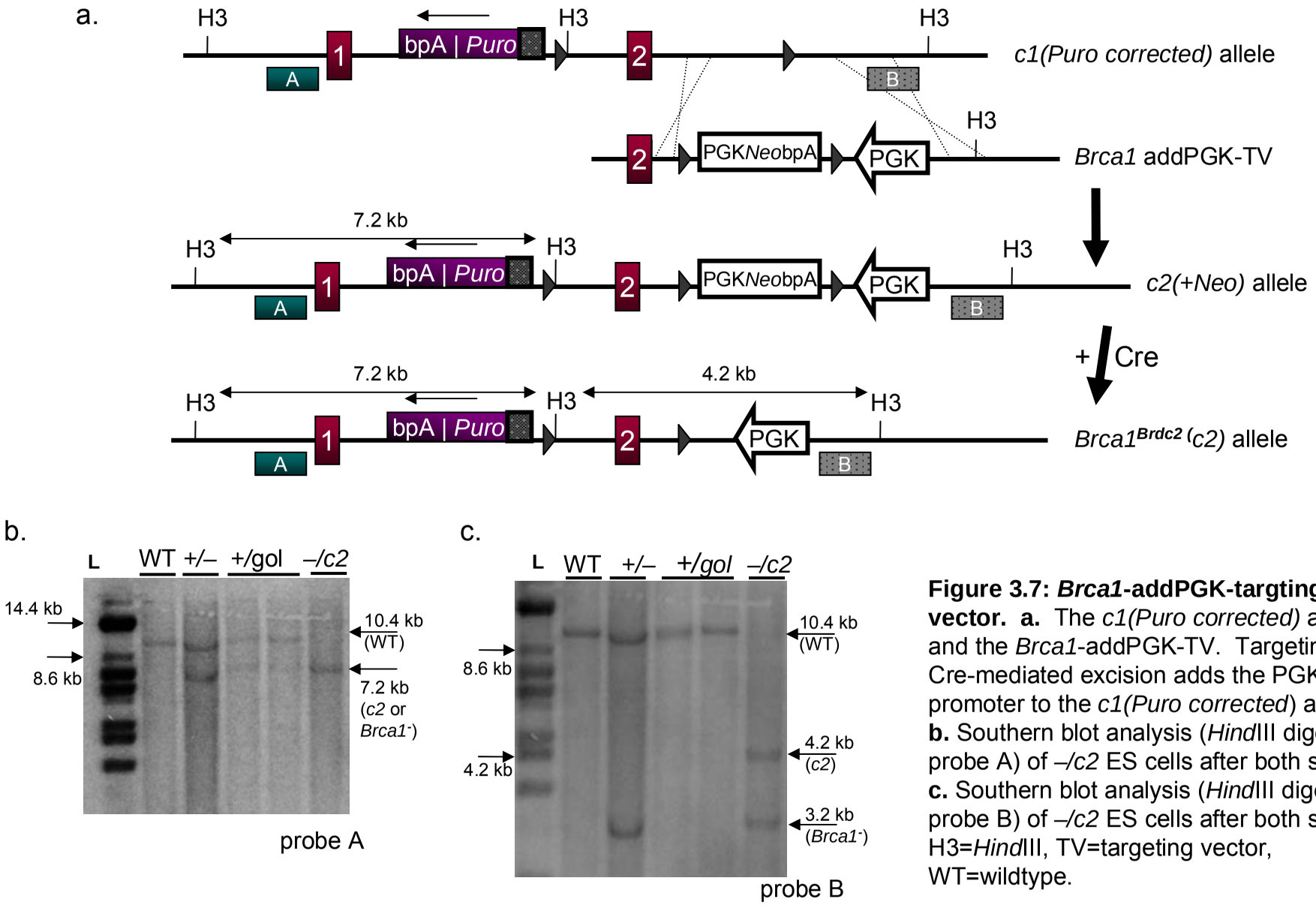


Figure 3.7: *Brca1*-addPGK-targting vector. a. The *c1(Puro corrected)* allele and the *Brca1*-addPGK-TV. Targeting and Cre-mediated excision adds the PGK promoter to the *c1(Puro corrected*) allele. **b.** Southern blot analysis (*Hind*III digest, probe A) of *–/c2* ES cells after both steps. **c.** Southern blot analysis (*Hind*III digest, probe B) of *–/c2* ES cells after both steps. H3=*Hind*III, TV=targeting vector, WT=wildtype.

selection cassette. Two *–/c2* ES cell lines were generated from these electroporations (Figure 3.7b and c).

3.2.2.4.1 The Puro cassette from the c2 allele is fully functional: *–/c2* ES cells were used to check for the percentage of background arising when the *c2* allele underwent Cre-mediated excision and cells were selected in HAT– (for the *Brca1*$^{-}$ allele) and puro– (for the excised *c2* allele) containing medium. Such cells were expected to be non-viable, so the percentage of HAT-resistant, puro-resistant colonies was expected to accurately reflect the background, which would be used in calculating how many ES cells to use in the suppressor screen.

Surprisingly, many HAT-resistant, puro-resistant colonies resulted after *–/c2* cells were electroporated with Cre. It seemed unlikely that the screen background was so high, as the selection criteria were fairly stringent. Instead, it seemed more likely that the *–/c2* ES cells used were a mixed population, and included either trisomic cells (carrying one wildtype allele, one *Brca1*$^{-}$ allele, and one *c2* allele) or cells with a secondary mutation which were viable despite the loss of *Brca1*.

3.2.2.4.2 –/c2 daughter cell lines do not differ in efficiency of Cre-recombination: To investigate possible secondary mutations in the *–/c2* ES cell line, two experiments were performed.

First, *–/c2* Es cells were plated at low density and five individual colonies were picked. Each was expanded and subjected to Cre-mediated excision of the *c2* allele and subsequent puro selection. If the original *–/c2* ES cell line was a mixed population (with a percentage of the cells either trisomic for *Brca1* or carrying a secondary mutation), the daughter cell lines were expected to segregate into two groups with either high or low/no viability following excision of the *c2* allele. However, each of five lines analyzed yielded a similar number of puro-resistant colonies (Table 3.1). The numbers were much lower than expected, as the Cre preparation used generally yielded a 10-30% excision efficiency. This result may have been due to suboptimal

Table 3.1: Plating efficiency of *–/c2* daughter cell lines following electroporation with Cre and puro selection.

Daughter cell line	Cells electroporated and plated	Total colonies	Plating efficiency (%)
1	8.8×10^6	1176	0.013
2	9.7×10^6	1950	0.020
3	9.6×10^6	2580	0.027
4	6.4×10^6	2460	0.038
5	8.8×10^6	1920	0.022
Average			0.024

electroporation conditions – the cells were quite dense at time of electroporation and were likely no longer in log-phase growth. However, while lower than expected, the five results did not differ appreciably from one another, suggesting that the original *-/c2* cell line was not a mixed population.

The original *–/c2* ES cell line was used in a second experiment to estimate the efficiency of Cre-mediated excision of the *c2* allele, this time using a fresh preparation of Cre. The efficiency of Cre excision of the *c2* allele in these cells was ~23%, within the expected 10-30% range (Table 3.2).

These two experiments showed that Cre-mediated excision of the *c2* allele occurs at the expected frequency, and that the *–/c2* ES cell line does not appear to have a segregating secondary mutation, although the possibility of secondary mutations being present is not completely ruled out.

3.2.2.4.3 –/c2 cells are not trisomic; the Brca1^{Brdm2} or gollum allele: To rule out trisomy at the *Brca1* locus in *–/c2* ES cells, these cells were subjected to electroporation with Cre and eighteen puro-resistant colonies were picked and expanded. Southern blot analysis of genomic DNA from these cells indicated clearly that the cells had just two alleles (Figure 3.8; 6 of the 18 are shown). Additionally, neither *–/c2* ES cells nor the five *–/c2* daughter cell lines described above exhibit trisomy on Southern analysis – a wildtype *Brca1* allele is not detected (Figure 3.8 and data not shown).

The excised form of the *c2* conditional allele is designated *Brca1^{Brdm2}*. As the allele is predicted to give rise to a protein which lacks part of the N-terminal RING domain, it is also known as *Brca1^{gollum}* or, in this work, as "*gol"* (Figure 3.3c).

3.2.2.4.4 –/gol ES cells do not produce full-length Brca1 mRNA: RT-PCR analysis of *–/gol* ES cell RNA using primers in exons 1 and 6 of *Brca1* indicated that no full-length *Brca1* transcript is produced in these cells (Figure 3.9a). The only RT-PCR product detected in *–/gol* samples is a product in which exon 1 is spliced to exon 3 (*Brca1 ΔX.2*). Sequence analysis shows

Table 3.2: Plating efficiency of *–/c2* parental cell line after electroporation with Cre and puro selection. The plating efficiency of *–/c2* cells electroporated with Cre were determined with or without puro selection. The estimated Cre efficiency was calculated by comparing the number of colonies resulting after puro selection to the number of colonies expected if no puro selection was used (as determined by the control experiment). The overall estimation of Cre efficiency was 23%.

Cells plated	Drug selection	Total colonies	Plating efficiency, no drug selection (%)	Expected number of colonies	Estimated Cre efficiency (%)
10^3	puro	10		71	14
10^4	puro	225		708	32
10^5	puro	1500		7080	21
10^4	none	708	7.08		

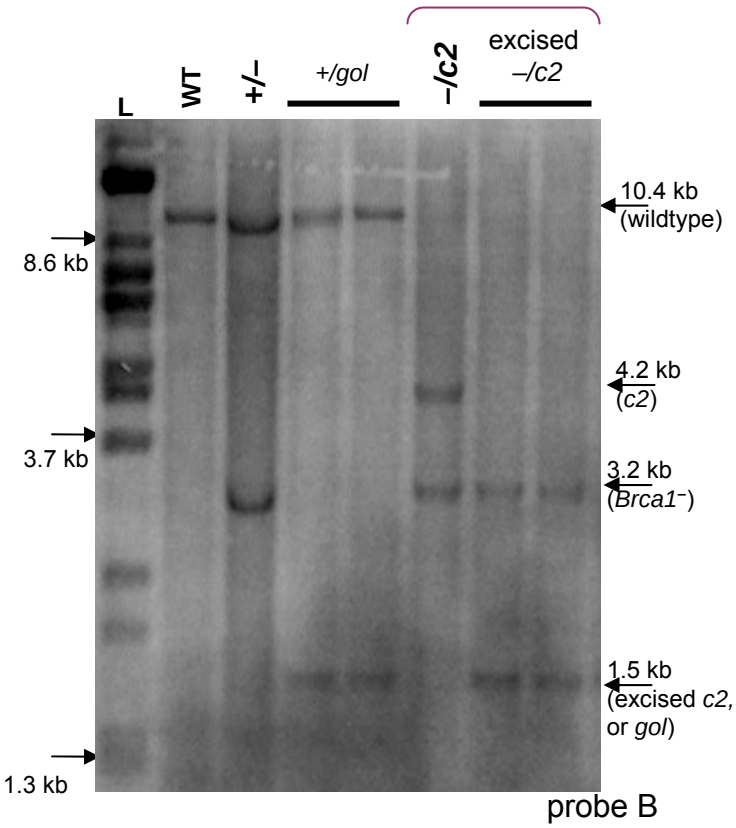


Figure 3.8: *–/c2* ES cells after Cre-mediated excision. Southern blot analysis (*Hind*III digest, probe B) of puro-resistant colonies resulting from Cre-mediated excision of the *c2* allele from *–/c2* ES cells. Genomic DNA from the parental *–/c2* ES cells is shown as a reference. WT=wildtype.

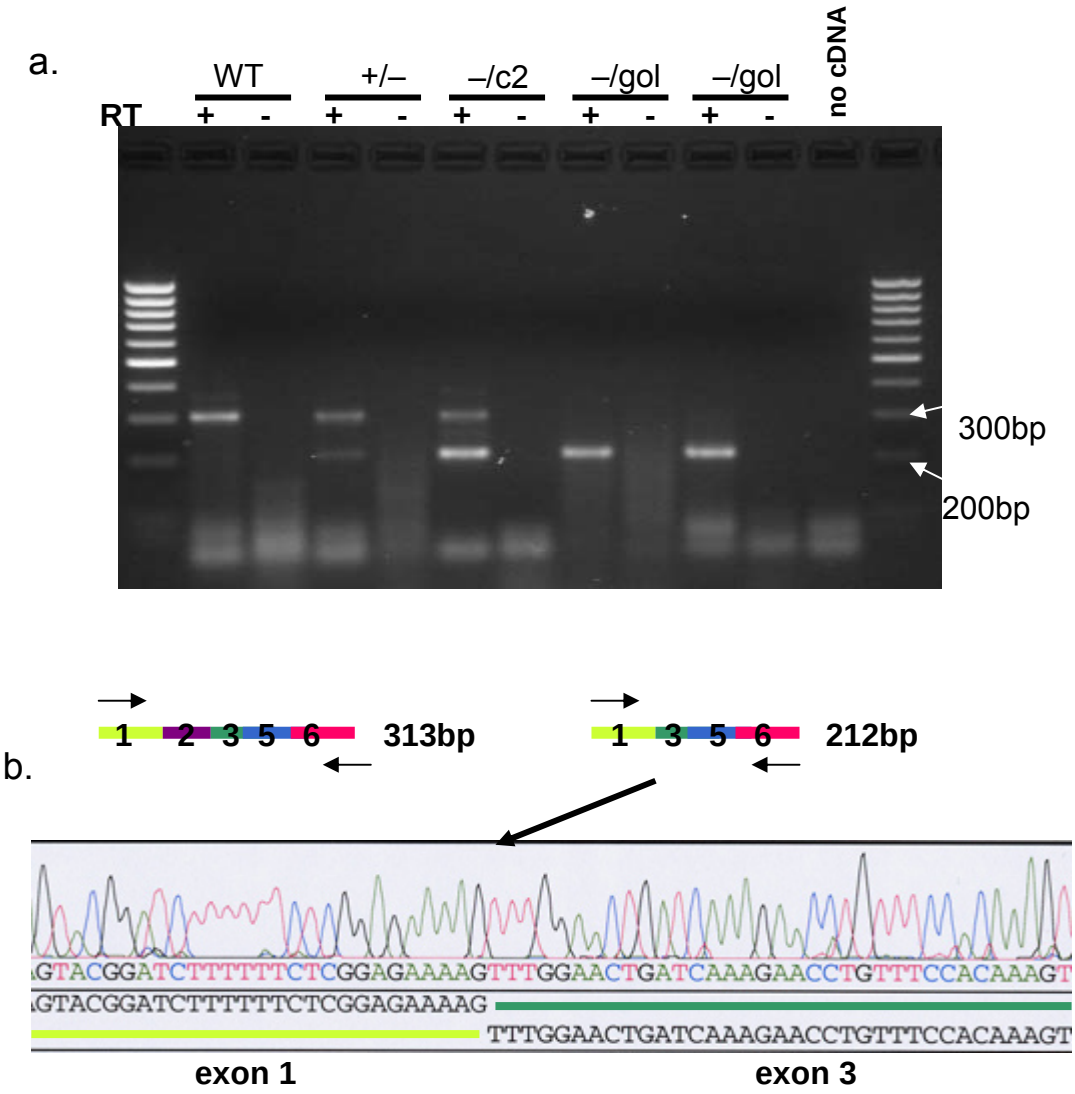


Figure 3.9: –/gol cells lack full-length *Brca1* mRNA. a. RT-PCT of wildtype, +/–, and *–/gol* cells reveals that while no full-length *Brca1* mRNA is produced (313 bp), a exon1-3 splice isoform (212 bp) is expressed in *–/gol* and +/– cells. **b.** Sequence analysis of the smaller product reveals a precise splice of exon 1 to exon 3. WT=wildtype.

that the exon 1 – exon 3 splice in *Brca1 ΔX.2* is precise (Figure 3.9b). This *Brca1 ΔX.2* product is also detected in *+/–* ES cells. Further analysis of the transcripts of the *gol* and *Brca1*$^{-}$ alleles will be described in Chapter 6.

3.2.2.5 gol/gol ES cells generated for further studies

gol/gol ES cells were generated by selecting for LOH in *–/gol* ES cells. The *Hprt* selection cassette in the *Brca1*$^{-}$ allele was selected against using 6-thio-guanine (6TG), which is metabolized by the *Hprt* gene product into a toxic base analogue. Mini-Southern analysis showed that 86% of 6TG-resistant colonies had lost the *Hprt* allele of *Brca1* and were presumably homozygous for the *gol* allele. Twelve of these *gol/gol* lines were expanded, and two (C11 and D9) were used in the studies detailed in Chapters 5 and 6 (Figure 3.10).

3.2.2.5.1 FISH analysis of gol/gol ES cells: Fluorescence in-situ hybridization (FISH) was performed on both C11 and D9 *gol/gol* ES cell lines by Ruby Banerjee of the Sanger Institute, using the mouse BAC RP23-210E12, which contains the entire *Brca1* genomic region as well as some surrounding sequence. This analysis was done to rule out a localized deletion of the *Brca1* region in these cells being selected instead of the desired LOH event. Figure 3.11 shows that both *gol/gol* cell lines carry two copies of the *Brca1* genomic locus. Wildtype cells also have two copies of this genomic locus (data not shown).

3.2.2.6 Brca1-gollum-TV

The viability of the *gol* allele made generation of a mouse carrying this allele desirable. However, attempts at blastocyst injection using either *–/gol* or *gol/gol* ES cells were unsuccessful and only resulted in low-percentage chimæras which did not transmit the allele through the germline. This was attributed to the numerous manipulations these cells had undergone in culture. Therefore, a final version of the conditional vector (*Brca1*-cond2-TV) was constructed which recapitulates the *c2* allele (Figure 3.3b). A Cre-excised derivative of the *Brca1*-cond2-TV gave *Brca1*-gollum-TV, which was used to generate *+/gol* ES cells (Figure 3.12a). One *+/gol* ES cell lines was

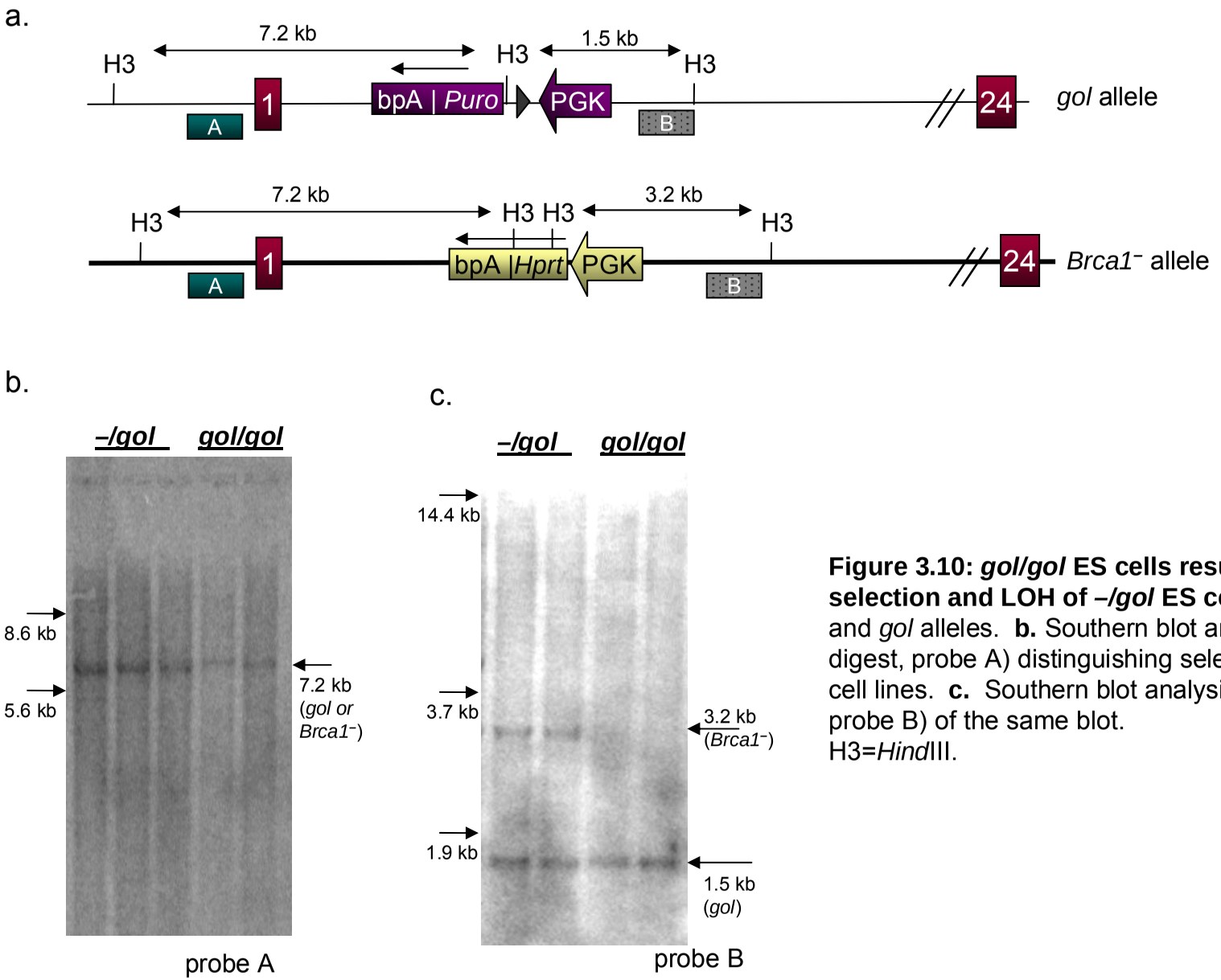
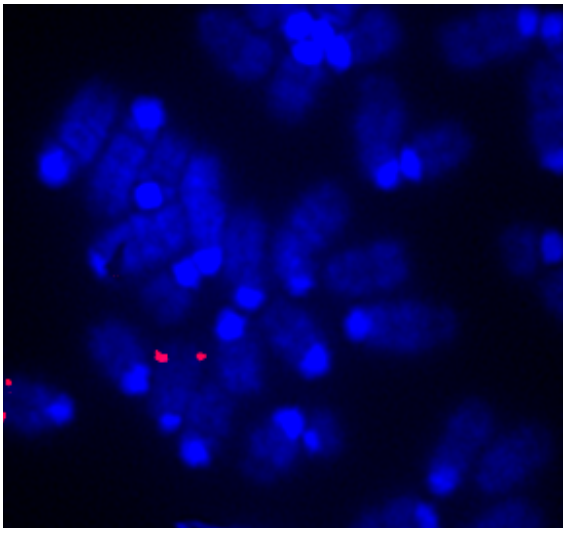
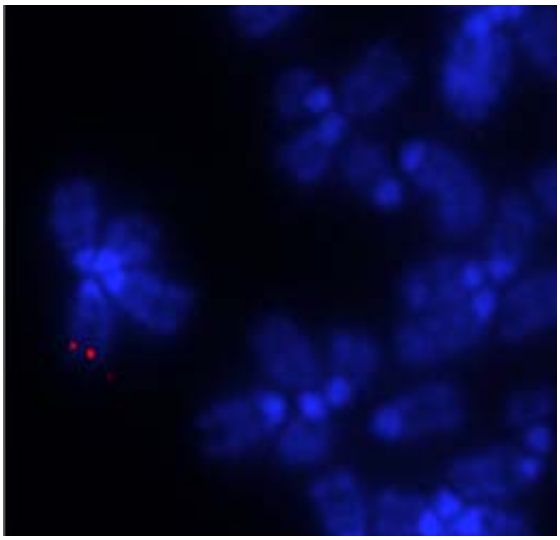


Figure 3.10: *gol/gol* ES cells resulting from 6TG selection and LOH of *–/gol* ES cells. a. *Brca1*⁻ and *gol* alleles. **b.** Southern blot analysis (*Hind*III digest, probe A) distinguishing selected and parental cell lines. **c.** Southern blot analysis (*Hind*III digest, probe B) of the same blot.
H3=*Hind*III.



gol/gol
(C11)



gol/gol (2)
(D9)

Figure 3.11: Fluorescent *in situ* hybridization shows that *gol/gol* ES cells have two copies of the *Brca1* genomic region. *gol/gol* ES cell lines were subjected to FISH using the mouse BAC RP23-210E12 (containing the entire *Brca1* genomic region and surrounding sequence, biotinylated by nick-translation). Following hybridization, the BAC was visualized by two layer detection with FITC-labeled antibodies. DAPI (blue) was used to stain chromosomes. FISH was performed by Ruby Banerjee of the Sanger Institute.

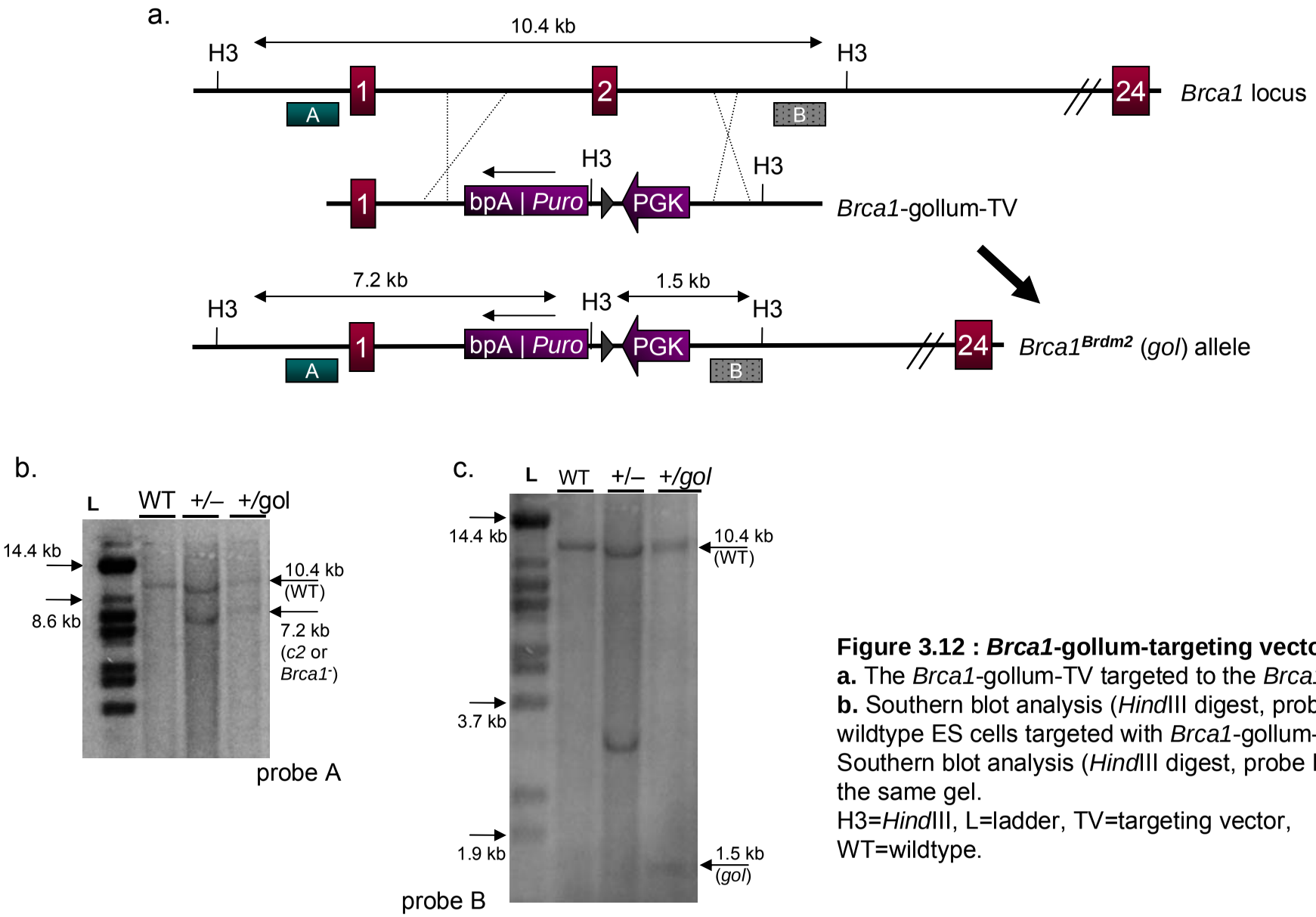


Figure 3.12 : *Brca1*-gollum-targeting vector.
a. The *Brca1*-gollum-TV targeted to the *Brca1* locus. **b.** Southern blot analysis (*Hind*III digest, probe A) of wildtype ES cells targeted with *Brca1*-gollum-TV. **c.** Southern blot analysis (*Hind*III digest, probe B) of the same gel.
H3=*Hind*III, L=ladder, TV=targeting vector, WT=wildtype.

isolated (Figure 3.12b and c), and generation of a mouse model using this cell line is in progress.

3.3 DISCUSSION

This chapter describes the generation of four novel alleles of mouse *Brca1* (Figure 3.3, targeting schematic shown in Figure 3.2). Each of the alleles generated for this study targets exon 2 of *Brca1*, which contains the translational start site. A previously-published report of an exon 2 *Brca1* knockout demonstrated that this allele behaves as a null allele: homozygous embryos and ES cells cannot be generated, and mice heterozygous for the mutation had no increased predisposition to tumourigenesis (Ludwig, 1997). The majority of published knockout alleles of *Brca1* target exon 11, which codes for a large portion of the protein. However, as the alleles generated in this study were specifically designed for use in a functional screen, the use of exon 11 as a target was not thought to be ideal. A natural *Brca1* splice isoform which lacks all of exon 11 (*Brca1* Δ*X.11*) is known to exist in both mice and humans, and is thought to have either partial –or different– function as compared to full-length *Brca1,* meaning it may not be an ideal mutation for use in a functional screen (Thakur, 1997; Wilson, 1997; Xu, 1999c; Bachelier, 2000).

Brca1^{-} behaves similarly to previously-published null alleles in that homozygous mutant ES cells cannot be generated (Hakem, 1996; Liu, 1996; Ludwig, 1997; Shen, 1998; Hohenstein, 2001). Two conditional alleles of *Brca1* were also generated in this study; the *c1* allele has been used primarily in tumourigenesis studies in mice, and will be described further in Chapter 4. The *c2* conditional allele was generated for use in a gene trap suppressor screen, but experimentation demonstrated that Cre-mediated excision of the *c2* allele generated a novel allele which did not behave as a null allele.

This novel allele lacks exon 2 and is termed *gollum* (*gol*) because it is predicted to give rise to a protein with a mutated or missing RING domain. ES cells carrying two copies of the *gol* allele (or one copy of *gol* and one copy

of *Brca1*$^{-}$) are viable and healthy. They do not appear to be trisomic and do not produce full-length *Brca1* mRNA. They do produce a splice isoform of *Brca1* (*Brca1 ΔX.2*) in which exon 1 is spliced precisely to exon 3 (Figure 3.9). The viability of *gol/gol* ES cells strongly suggests that this isoform is functional. The loss of exon 2 means that any potential Brca1 protein produced from the *gol* allele will lack part of the highly-conserved N-terminal RING domain, thus suggesting that this domain is not required for normal growth in culture.

The *Brca1 ΔX.2* isoform was also detected in +/– cells, but not in wildtype cells, which suggests that this isoform is also produced by the *Brca1*$^{-}$ allele, despite the difference in viability between the *gol* and *Brca1*$^{-}$ alleles. The functionality of the *Brca1 ΔX.2* isoform will be discussed further in Chapter 6.

Only one other group has described a double-targeted *Brca1* ES cell line (Table 1.3 #5). In their case, only one cell line was recovered after screening numerous clones (Gowen, 1996), indicating that this is a rare event. This allele appears to give rise to a protein lacking exon 11, mimicking the natural *Brca1* Δ*X.11* splice isoform. There is a possibility that secondary mutations which compensate for the loss of *Brca1* are present in this cell line, but retargeting a wildtype copy *Brca1* into one of the mutant loci rescued the mutant phenotype, suggesting that any secondary mutation is likely to be independent of Brca1-related pathways (Moynahan, 2001). A second group has reported a homozygous mutant mouse which carries a truncated version of Brca1 (Table 1.3 #9), and a few groups have generated MEF lines homozygous for a *Brca1* mutant allele (although these lines often additionally carry a *p53* mutation) (Shen, 1998; Xu, 1999c; Ludwig, 2001). While secondary mutations in the *–/gol* or *gol/gol* cell lines cannot be ruled out, puro-resistant colonies are recovered at the expected frequency following Cre-mediated excision of *–/c2* cells (Table 3.2). Additionally, analysis of the *–/c2* daughter cell lines suggested that, based on the number of puro-resistant colonies resulting after Cre-mediated excision of *c2*, a segregating secondary mutation was not present in the original *–/c2* cell line (Table 3.1).

The surprisingly viability of the *gol* allele precludes the use of *–/c2* cells in the suppressor screen originally planned, but provides a unique opportunity to study the consequences of ablation of a specific domain of *Brca1*. In the following chapters, the response of *gol/gol* cells to DNA damage and the molecular characterization of the *gol* allele will be described.