CHAPTER FOUR:

MOUSE MODELS

4.1 INTRODUCTION

Both the *Brca1*$^{-}$ and *c1* alleles generated for use in the conditional ES cell system discussed in Chapter 3 were additionally used to generate mouse models to study *Brca1*-related tumourigenesis *in vivo*.

As mice heterozygous for *Brca1* knockout alleles generated in previously-published studies do not appear to have an increased predisposition to tumourigenesis (Table 1.3), *Brca1*$^{+/-}$ mice generated in this study were crossed with mice deficient for the Bloom's Syndrome gene *Blm*. The *Blm* gene product is a RecQ-like helicase which is involved in resolving recombination intermediates. Loss of this gene results in an elevated rate of mitotic recombination and subsequent loss of heterozygosity (LOH) (reviewed in Hickson, 2003). A previous study using *Blm*$^{-/-}$ mice showed that 29% of *Blm*-deficient mice developed a wide range of tumours by twenty months of age, and that a *Blm*-deficient background accelerated tumourigenesis in *Apc*$^{min/+}$ mice. Tumours resulting in these *Apc*$^{min/+}$, *Blm*$^{-/-}$ mice had lost the wildtype *Apc* allele. It was expected that the *Blm* deficiency would mediate LOH at the *Brca1* locus, and thus accelerate tumourigenesis (Luo, 2000). However, similar to what has previously been observed in mice heterozygous for other *Brca1* knockout alleles, mice carrying the *Brca1*$^{-}$ allele do not seem predisposed to tumourigenesis, even on a *Blm*-deficient background.

Mice carrying the *c1* conditional allele were also generated, and crossed to mice carrying a Cre transgene. As the *Brca1*-related tumour profile in the mouse may differ from that of the human, Cre was expressed under the control of either a breast-specific promoter (*β-casein*-Cre) or the ubiquitously-expressed cytomegalovirus promoter (CMV-Cre). Similar to mice carrying the *Brca1*$^{-}$ allele, neither *Brca1*$^{-/c1}$ nor *Brca1*$^{c1/c1}$ mice carrying a Cre transgene appear to be predisposed to tumourigenesis.

Mice carrying the *gol* allele, the other novel replacement allele from this study, are in the process of being generated.

4.2 RESULTS

4.2.1 The *Brca1*$^{-}$ allele

4.2.1.1 Germline transmission of the Brca1^{-} *allele*

Two +/– ES cell clones (C2 and D2) were injected into blastocyst-stage embryos for the generation of *Brca1* knockout mice. Chimæras resulting from both injections successfully transmitted the *Brca1*$^{-}$ allele through the germline.

One hallmark of the majority of the previously described *Brca1* knockout alleles is embryonic lethality of homozygous mutant animals (Hakem, 1996; Liu, 1996; Ludwig, 1997; Shen, 1998; Hohenstein, 2001). Of all 593 progeny from heterozygous intercrosses (*Brca1*$^{+/-}$ X *Brca1*$^{+/-}$) genotyped to date, no homozygous mutant (–/–) animals have been identified: 206 (34.7%) were wildtype (+/+), and 387 (65.3%) were heterozygous (+/–). These numbers correspond well with the 1 (+/+) : 2 (+/–) ratio of progeny expected from such a cross if –/– animals do not survive. The precise timing of embryonic lethality was not determined, but the targeting vector used to generate the *Brca1*$^{-}$ allele was derived from the vector used by Ludwig *et al.* (Table 1.3 #1), and their homozygous mutant animals did not survive beyond E9.5. The inability to recover homozygous mutant animals indicates that the *Brca1*$^{-}$ allele from this study behaves like previously-published null alleles of *Brca1*.

4.2.1.2 Tumourigenesis study of Brca1$^{+/-}$ *mice in conjunction with a Blm mutation (Houston)*

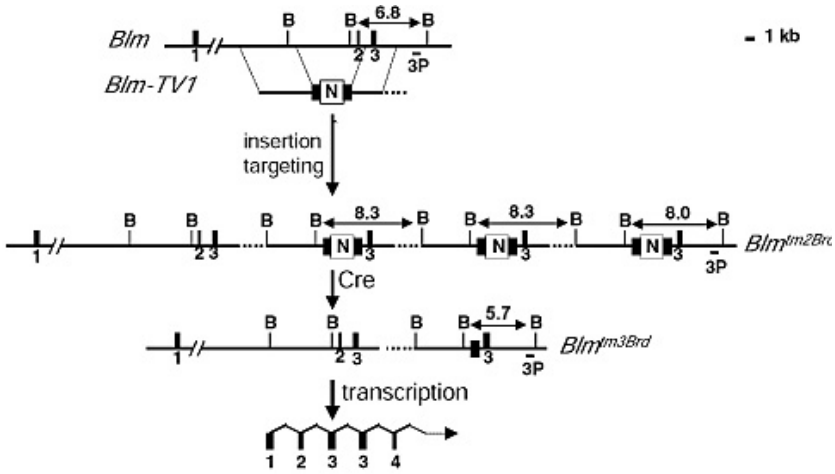
Earlier studies have indicated that mice carrying a *Brca1* knockout allele do not have an increased predisposition to tumourigenesis compared to wildtype mice. Several groups have used secondary mutations to try and accelerate *Brca1*–related tumourigenesis, most commonly by crossing the mutation onto a *p53*$^{+/-}$ or *p53*$^{-/-}$ background (Cressman, 1999b; Hohenstein, 2001). For this study, it was decided to cross the *Brca1*$^{+/-}$ mice onto a *Blm*-deficient

background (Figure 4.1 shows genotyping information for this gene). The *Blm* gene product is a RecQ helicase homologue involved in resolving recombination intermediates (Ellis, 1995; Hickson, 2003). Loss of *Blm* leads to a higher rate of mitotic recombination and subsequent LOH. *Blm* knockout mice generated by a colleague, Guangbin Luo, were crossed to mice carrying the *Brca1*$^{-}$ allele. Guangbin has previously shown that *Blm*-deficient ES cells have an accelerated rate of mitotic recombination, about 18-fold greater than that of wildtype cells (Luo, 2000). As *Brca1* is a caretaker gene, its loss is likely not the rate-limiting step in tumourigenesis, and it was hoped that tumourigenesis could be accelerated by early LOH at the *Brca1* locus in *Brca1*$^{+/-}$, *Blm*$^{-/-}$ mice.

An earlier study by colleagues Irma Santoro and Guangbin Luo indicated that a *Blm*-deficient background accelerated tumourigenesis in *Apc*$^{min/+}$ mice. Genotyping of tumour tissue from *Apc*$^{min/+}$, *Blm*$^{-/-}$ mice indicated that tumours had lost the wildtype allele of *Apc* (Luo, 2000). *Blm*$^{-/-}$ mice, like mice carrying a *p53* mutation, are themselves tumour-prone, but less so than are *p53* mutants (29% of *Blm*$^{-/-}$ animals develop tumours by 20 months, compared to 50% by ~18 months for *p53*$^{+/-}$ mice or ~4.4 months for *p53*$^{-/-}$ mice (Donehower, 1995; Luo, 2000)), meaning that *Brca1*-related tumourigenesis may have more time to become apparent on the *Blm*-deficient background than on a *p53*-deficient background.

Four cohorts of mice were generated for this tumourigenesis study, consisting of virgin and mated *Brca1*$^{+/-}$, *Blm*$^{+/-}$ and *Brca1*$^{+/-}$, *Blm*$^{-/-}$ female mice. Animals were still being generated when a flood in Houston in June of 2001 killed many of the mice in the study. At that time, most animals in the experimental *Brca1*$^{+/-}$, *Blm*$^{-/-}$ cohort were still <1 year of age, and no tumours had been observed in any animals. As the Bradley lab had by this time moved to England, the decision was made not to continue this study, and many of the remaining mice were used for embryo rederivation to transport the alleles over to the lab in England.

a.



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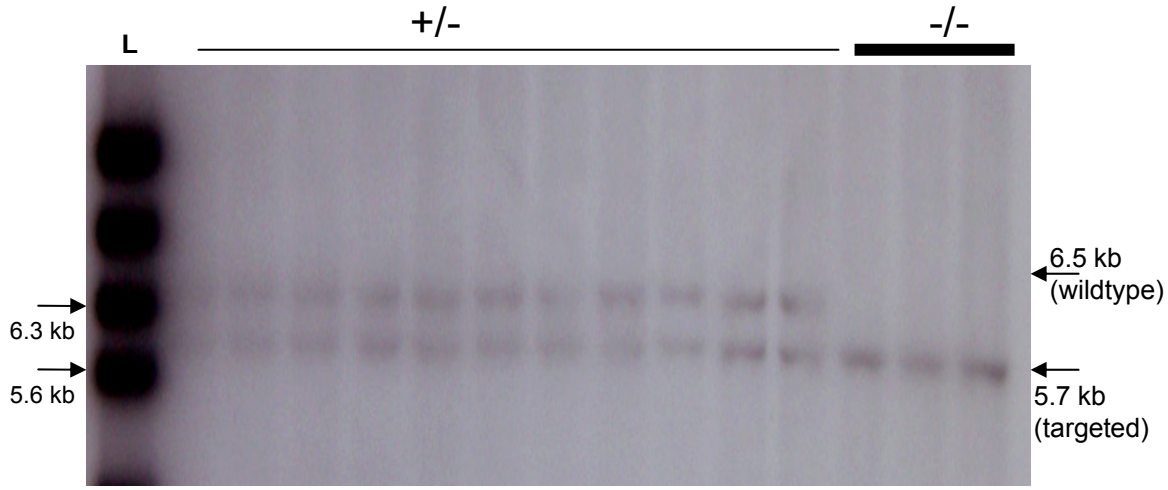

Figure 4.1: *Blm* locus genotyping. a. Blm targeting vector schematic. **b.** Southern blot analysis of *Bam*HI-digested mouse genomic DNA probed with the *Blm* genotyping probe.
L=ladder. Targeting vector figure modified from (Luo, 2000).

4.2.1.3 Whole-mount analysis of mammary glands from Brca1$^{+/-}$, Blm mice

Once embryo rederivation was completed, the remaining animals were passed to Dr. Daniel Medina from Baylor College of Medicine who performed whole-mount analysis of mammary glands from *Brca1*$^{+/-}$, *Blm*$^{+/-}$ or *Brca1*$^{+/-}$, *Blm*$^{-/-}$ female mice, aged 15-16 months (n=5) or 22-24 months (n=7). In the 15-16 month old mice, the mammary gland was normal in development and involution and no lesions were identified, either premalignant or malignant. In the older animals, the glands likewise had normal morphology with no signs of tumours, though one gland had a small (<2 mm) hyperplastic, squamous alveolar lesion. No further analysis was done on the mice from the Houston tumourigenesis study. Figure 4.2 shows a representative mammary gland from younger mice of each genotype.

4.2.1.4 Tumourigenesis study: Brca1$^{+/-}$, Blm$^{-/-}$ (England)

A second *Brca1*$^{-}$ tumourigenesis study was set up in England using a cohort of 57 *Brca1*$^{+/-}$, *Blm*$^{-/-}$ female and 50 *Brca1*$^{+/-}$, *Blm*$^{-/-}$ male mice. At time of writing, the animals are 15-22 months of age. Although some animals have died of natural causes or been terminated due to unrelated illness, only three mice have developed discernable tumours: one lung tumour (in a 12 month old female), a tumour of unknown origin on the shoulder (in a 12.3 month old male), and a neck tumour (in a 18.4 month old male). The wildtype allele of *Brca1* can be detected in tissue from all tumours tested, indicating that tumours resulting in these mice are more likely to be due either to natural causes or from loss of the *Blm* gene product (Figure 4.3, lanes 2 and 3). Figure 4.4 shows a Kaplan-Meier survival analysis of these animals compared to *Blm*$^{+/-}$ or *Brca1*$^{+/-}$ control animals; the difference is not significant (in a previous study in this lab, *Blm*$^{+/-}$ mice had lifespans not significantly different from those of wildtype mice). The oldest of these mice are not yet two years old, so it is still possible that additional tumours will be observed over the next several months. However, tumourigenesis resulting from the *Blm* deficiency is also expected to become more apparent over the next few months.

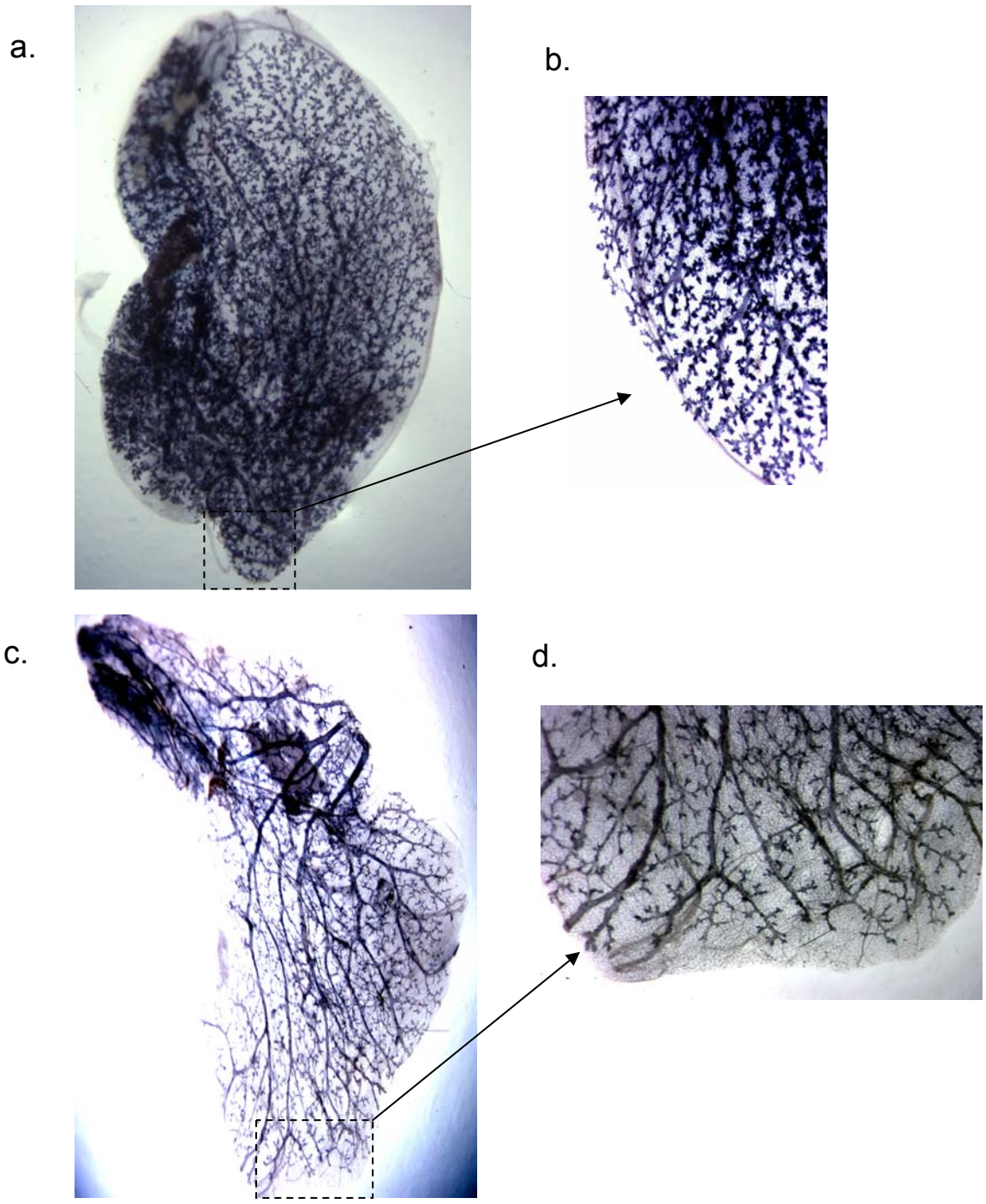

Figure 4.2: Whole-mount analysis of mammary glands from *Brca1*$^{+/-}$*; Blm*$^{-/-}$ *or Brca1*$^{+/-}$*; Blm*$^{+/-}$ mice. **a.** A mammary gland from a 16 month old, female *Brca1*$^{+/-}$*, Blm*$^{-/-}$ mouse. **b.** Same sample, close-up to show normal branching structure of ductal tree. **c.** A mammary gland from a 15 month old female *Brca1*$^{+/-}$*, Blm*$^{+/-}$ mouse. **d.** Same sample, close-up to show normal branching structure of ductal tree. Both mice had undergone multiple pregnancies.

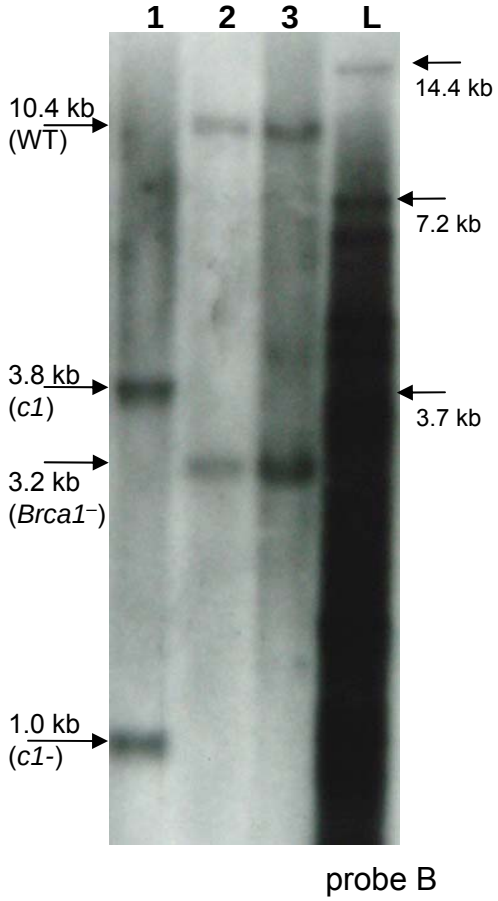

Figure 4.3: Tumours have not lost the wildtype allele of *Brca1*.
Southern blot analysis (*Hind*III digest, probe B) of tumours from a *c1/c1*– mouse (lane 1), and two tumours from *Brca1*$^{+/-}$, *Blm*$^{-/-}$ mice (lanes 2 and 3).
L=ladder, WT=wildtype.

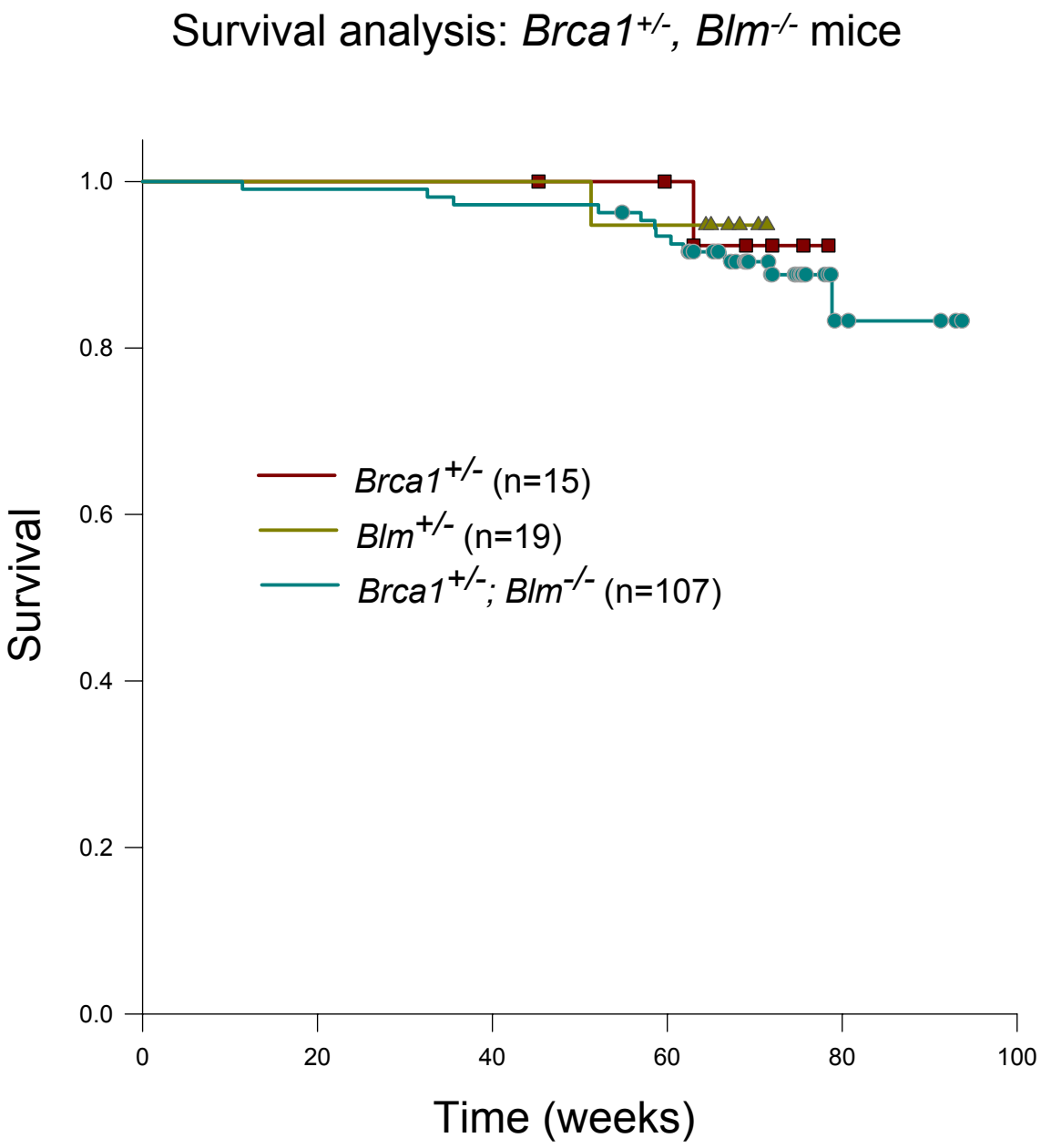

Figure 4.4: Survival analysis of *Brca1*$^{+/-}$, *Blm*$^{-/-}$ mice. Kaplan-Meier survival analysis of *Brca1*$^{+/-}$, *Blm*$^{-/-}$ and *Brca1*$^{+/-}$ and *Blm*$^{+/-}$ mice. Mice dying of natural causes, or terminated because of illness or tumourigenesis were all included. Only three *Brca1*$^{+/-}$, *Blm*$^{-/-}$ mice with tumours have been identified.

These data indicate that the *Brca1*¯ allele generated in this study behaves similarly to previously-described knockout alleles in that heterozygotes do not have an increased predisposition to cancer by a mean age of 18 months, even on a background designed to accelerate tumourigenesis.

4.2.2 The *Brca1* conditional allele *c1* and tumourigenesis studies of mice carrying the *c1* allele

4.2.2.1 Germline transmission of the c1 allele

Two -/*c1* ES cell clones (D9 and F2) were injected into blastocyst-stage embryos for generation of conditional *Brca1* mice. Chimæras resulting from injection of the F2 clone successfully transmitted the *c1* allele through the germline.

4.2.2.2 Expression of Cre recombinase in the c1 tumourigenesis study

When this project began, a single study describing a conditional allele of *Brca1* had been published (Xu, 1999b). This allele was used for studying tumourigenesis in conjunction with Cre transgenes expressed from breast-specific promoters (Table 1.3 #8). Cre expression was driven either by the promoter from the gene encoding whey acidic protein (WAP), a milk protein expressed in mammary epithelium during pregnancy and lactation (Piletz and Ganschow, 1981; Robinson, 1995), or the MMTV–LTR (mouse mammary tumour virus–long terminal repeat), which is expressed in breast epithelium and ductal cells (Wagner, 1997). Very few tumours developed in these mice, even following a long latency, and complete loss of *Brca1* did not appear to have occurred in most tumours (Xu, 1999b).

Based on these results, it was considered worthwhile to investigate *Brca1*–related tumourigenesis in non-breast tissues of conditional mice (as well as in the mammary gland), in an attempt to determine if the tumour spectra of mice and humans differ. The use of a ubiquitously-expressed Cre transgene circumvents the problem of having Cre expressed mainly during lactation by milk-protein gene promoters. The disadvantage to using promoters of milk-protein genes is that while they are expressed specifically in mammary

tissues, they tend to be expressed in terminally-differentiated cells destined to die during involution of the mammary gland. Such cells are not believed to be the primary sites of tumour initiation (Rijnkels and Rosen, 2001; Smalley and Ashworth, 2003).

Mice carrying the *c1* allele were intercrossed, or crossed to *Brca1*$^{+/-}$ mice, then mated with mice carrying one of the Cre transgenes to generate the cohorts described in Table 4.1. Colleagues Hong Su and Xiaozhong Wang generated a mouse line carrying a Cre transgene expressed ubiquitously under the control of the cytomegalovirus (CMV) promoter. This transgene was knocked into the *Hprt* locus on the X-chromosome (Su, 2002). To generate a model which excised *Brca1* predominantly in the mammary gland, a Cre transgene knocked in at the *β-casein* locus (generated by colleagues Guangbin Luo and Yue He) was used. Previous studies have indicated that *β-casein* is expressed mainly in epithelial cells of the alveoli (at low levels in virgin mice and higher levels in pregnant/lactating animals), and in ductal cells at low levels in pregnant/lactating mice, with overall heterogeneous expression (Robinson, 1995). Mice lacking the *β-casein* gene are viable and are able to nurse their pups (Kumar, 1994).

Both types of Cre appeared to efficiently excise the *c1* allele in male and female mice (Figure 4.5 shows genotyping information for mice carrying the *β-casein*-Cre transgene; mice carrying CMV-Cre did not differ), and the excised conditional allele can be passed through the germline. The excised *c1* conditional allele is referred to in this work as "*c1*." Expression of *β-casein*-Cre was less breast-specific than expected, as excision of the *c1* locus was detected in tail-tip genomic DNA samples from first-generation mice (Figure 4.5c). This non-specific excision may be due to expression of the transgene during embryogenesis, although this was not determined.

4.2.2.3 Tumourigenesis study of mice carrying the c1 allele

A small *c1* tumourigenesis study encompassing a range of genotypes (Table 4.1) is in progress. At time of writing, the mice in this study are 13-19 months old. Although some mice have been removed from the study due to death

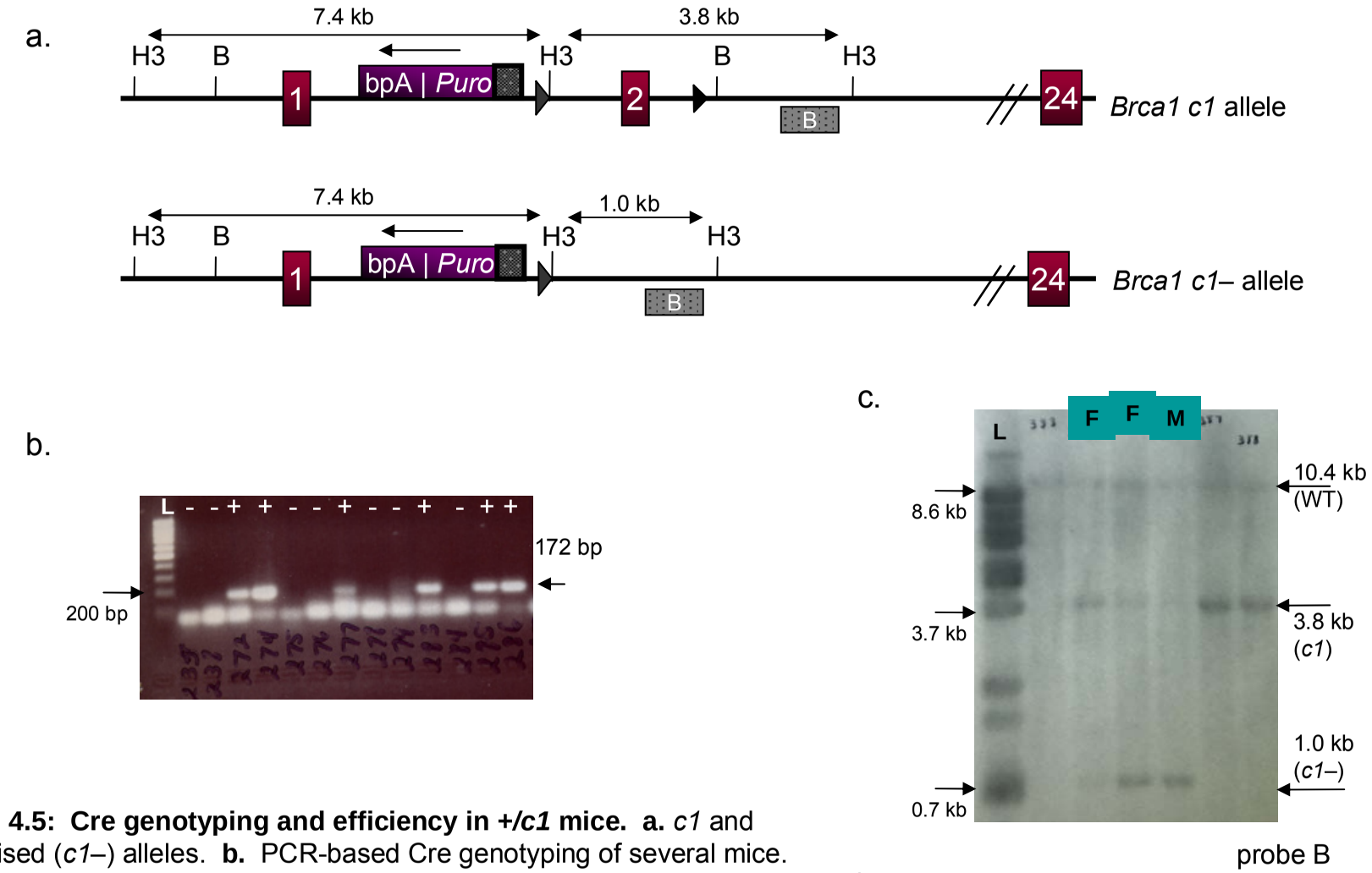

Figure 4.5: Cre genotyping and efficiency in +/*c1* mice. **a.** *c1* and *c1* excised (*c1*–) alleles. **b.** PCR-based Cre genotyping of several mice. A 172 bp product ("+") indicates the presence of Cre. **c.** *β-casein*-Cre, *Brca1*$^{+/c1}$ mice. Southern blot analysis (*Hind*III digest, probe B) indicates that Cre-mediated excision of exon 2 occurs in both male (M) and female (F) mice. H3=*Hind*III, L=ladder, WT=wildtype.

Table 4.1: Conditional (*c1*) tumourigenesis study.
See also Figure 4.6. Only one tumour has been identified in these mice to date.

Genotype	Total #	Cre allele		Number		Age of mice (weeks)	
		CMV	*β-casein*	Male	Female	Oldest	Youngest
+/c1	32			15	17	80	52
+/c1 with Cre	27	23	4	14	13	80	59
–/c1	5			5		73	61
–/c1 with Cre	5	5			5	70	54
c1/c1	12			6	6	77	52
c1/c1 with Cre	7	7		6	1	77	52
c1/c1–	9			4	5	79	59
c1/c1– with Cre	8	8		1	7	79	58

"*c1–*" is the excised version of the *c1* conditional allele. Excision occurred in the previous generation and the allele was passed on by the parent.

from natural causes, only one animal with a discernable tumour has been identified, a 13-month-old *c1/c1-* female with a mass on her shoulder. Genotyping of tissue from this tumour (Figure 4.3, lane 1) indicates that the unexcised *c1* allele has not undergone recombination and therefore should still be a wildtype allele. It is possible that more tumours will be observed in these mice over the next year, but at present, the single tumour cannot be used to draw any meaningful conclusions. Figure 4.6 shows the survival curves for mice carrying the *c1* allele; they do not differ significantly from the *Brca1*$^{+/-}$ or *Blm*$^{+/-}$ animals used as controls.

4.2.3 The *gol* allele

4.2.3.1 Germline transmission of the gol allele

Germline transmission of the *gol* allele was first attempted using *-/gol* (clones L5 and L7) or *gol/gol* (clones C11 and D9) ES cells. However, blastocyst injections of these clones generated only low-percentage chimæras which did not transmit the *gol* allele through the germline. This was likely due to the number of manipulations these cells had undergone. Chapter 3 described the generation of the *Brca1*-gollum-TV and *+/gol* ES cells. One *+/gol* ES cell clone (H8) has been injected for transmission of the allele through the germline, but this has not yet been achieved.

4.3 DISCUSSION

4.3.1 Tumourigenesis studies

4.3.1.1 The Brca1^{-} allele and tumourigenesis

Once mice carrying the *Brca1*$^{-}$ and *c1* alleles were successfully generated, they were used in tumourigenesis studies. Consistent with what has been observed in previous studies, mice heterozygous for the *Brca1*$^{-}$ allele do not have an increased predisposition to tumourigenesis before the age of eighteen months, even on a *Blm*-deficient background designed to accelerate tumourigenesis (Hakem, 1996; Liu, 1996; Ludwig, 1997; Shen, 1998; Hohenstein, 2001). Based on the results of the tumourigenesis study, the

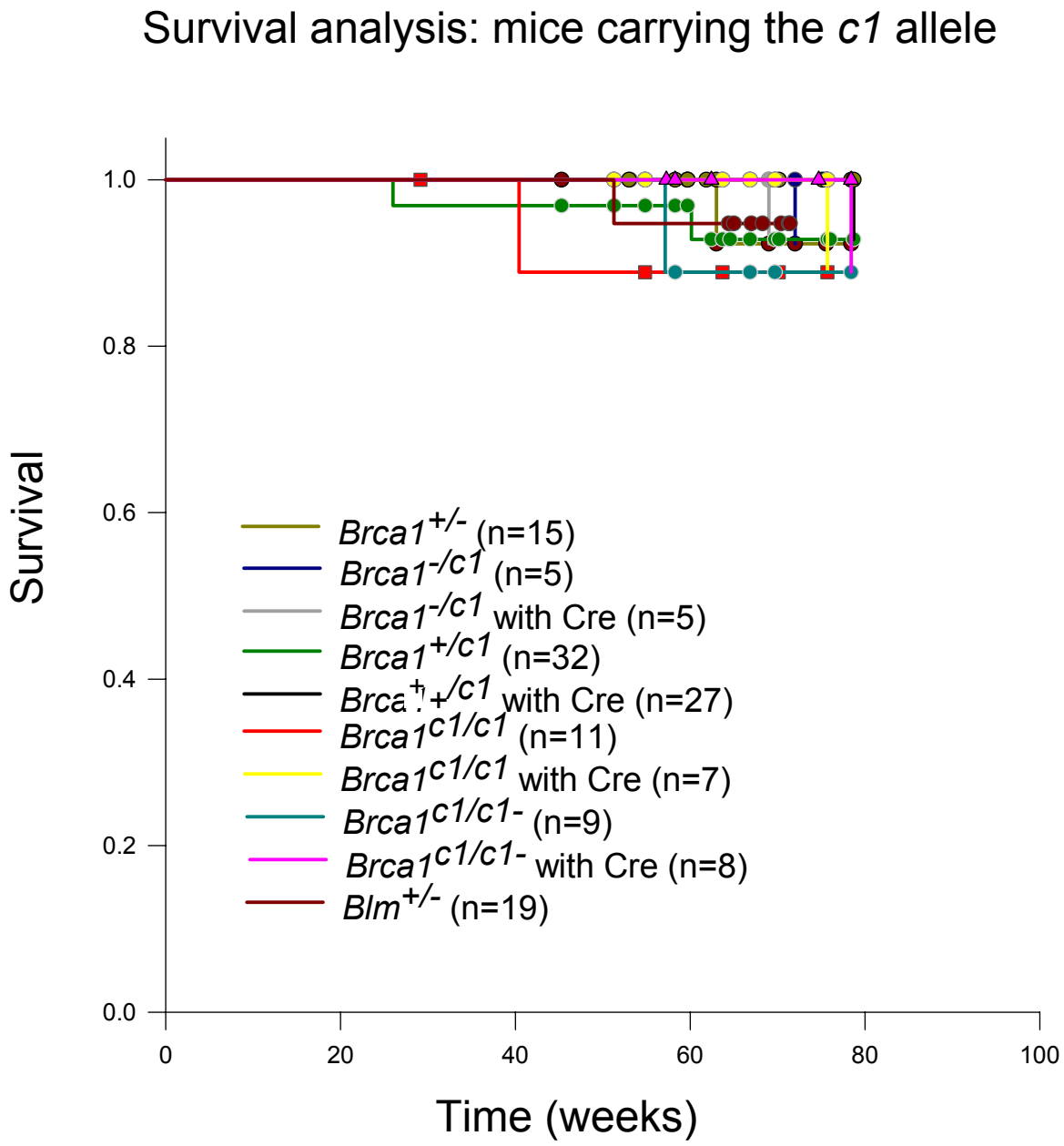

Figure 4.6: Survival analysis of mice carrying the *c1* allele. Kaplan-Meier survival analysis of mice carrying the *c1* allele with or without a Cre transgene. "Cre" in this figure means either CMV-Cre or *β-casein*-Cre; survival times for mice carrying these two transgenes did not differ. *Brca1*$^{+/-}$, *Brca1*$^{+/-}$ with Cre, and *Blm*$^{+/-}$ mice are used as controls. Mice dying of natural causes, or terminated because of illness or tumourigenesis were all included. Only one *Brca1*$^{c1/c1-}$ mouse with a tumour has been identified.

inability to recover viable –/– animals, and the inability to generate double-targeted (–/–) ES cells (discussed in Chapter 3), the *Brca1*$^{-}$ allele generated in this study behaves similarly to previously-described null knockout alleles of *Brca1*.

Southern blot analysis of the tumours isolated from *Brca1*$^{+/-}$, *Blm*$^{-/-}$ animals indicates that the wildtype allele of *Brca1* is still present in the tumours, although there does appear to be a decrease in the intensity of the wildtype band in at least one tumour (Figure 4.5, lane 3), perhaps due to contamination of the tumour sample with surrounding normal tissue. As *Brca1* is a caretaker gene, its loss is expected to result in genomic instability which should eventually result in other mutations, leading to tumourigenesis. The *Blm*-deficient background was utilized to try and make LOH at the *Brca1* locus an early event – but the tumours observed so far are more likely to be linked to the *Blm*-deficiency than the *Brca1* mutation. This supposition is based on previous observations that the tumour incidence in *Blm*$^{-/-}$ mice is ~3% before the age of one year. Of the 107 mice in the *Brca1*$^{+/-}$, *Blm*$^{-/-}$ tumourigenesis study, two mice (~2%) presented with tumours by the age of 12 months, close to the expected number (Luo, 2000).

Data gathered from *Brca1*$^{+/-}$, *Blm*$^{-/-}$ mice contrast with data from a previous study in which *Apc*$^{+/min}$, *Blm*$^{-/-}$ mice displayed accelerated polyp formation and tumourigenesis compared to either *Apc*$^{+/min}$ or *Blm*$^{-/-}$ mice. Analysis of tumours from *Apc*$^{+/min}$, *Blm*$^{-/-}$ mice indicated that LOH at the *Apc* locus had occurred. Loss of the wildtype allele of *Apc* in these tumours was presumably due (at least in part) to the *Blm* mutation (Luo, 2000). However, *Apc*$^{+/min}$ mice normally develop polyps by the age of 3 months, suggesting that an *Apc* mutation may be more of a rate-limiting factor in tumourigenesis than is a *Brca1* mutation (Luongo, 1994). It is possible that additional tumours will be observed in the *Brca1*$^{+/-}$, *Blm*$^{-/-}$ mice in the future. It is additionally possible that loss of both *Blm* and both copies of *Brca1* is a cell-lethal event, which would preclude tumourigenesis of such cells.

Several recent studies suggest that the loss of *Brca1* is not a rate-limiting step in tumourigenesis. Ludwig *et al.* demonstrated that mice homozygous for a C-terminal truncated allele, while viable, are prone to tumours (Table 1.3 #9). However, these tumours occurred with a mean latency of 1.4 years, and appeared to carry secondary mutations (Ludwig, 2001). Jonkers *et al.* used a *Brca1* and *p53* co-conditional mouse model to study breast tumourigenesis in conjunction with a *Keratin 14*–Cre transgene (Table 1.3 #10). They found that loss of *Brca1* was not necessary for tumour formation, although complete loss of *Brca1* could accelerate tumourigenesis in mice which had also lost *p53* (Jonkers and Berns, 2003). Both studies support the idea that *Brca1* is a caretaker gene which may be involved in, but is not sufficient for, the tumourigenic process.

4.3.1.2 The c1 allele and tumourigenesis

The conditional allele *c1* was also used to generate a mouse model to study *Brca1*–related tumourigenesis in conjunction with mice carrying either a breast-specific or ubiquitously-expressed Cre transgene. To date, only one tumour has been observed in the mice from this study. It is expected that, as for the *Blm-Brca1* tumourigenesis study above, a proportion of these mice will develop tumours after a longer latency and in conjunction with secondary mutations.

The promoters driving expression of the Cre transgenes used in this study were intended to be either ubiquitously expressed or expressed predominantly in the mammary gland. The use of CMV-Cre for ubiquitous expression of the Cre transgene was expected to circumvent the problems arising from the use of milk-protein promoters for Cre expression. It was possible that using a strong promoter for Cre expression would be detrimental, resulting in lethality of *Brca1*$^{-/c1}$ animals due to extensive excision. It cannot be ruled out that this happened in some animals or embryos, but a decrease in birth rates of potentially susceptible genotypes was not observed. Furthermore, previous and subsequent studies have shown that although this CMV-Cre transgene is strongly expressed, excision of a given transgene does not occur in every cell (Mills, 2002; Su, 2002).

Indeed, this was observed in our animals, in which a variable amount of *Brca1* excision was detected in tail-tip genomic DNA samples (Figure 4.5 and data not shown).

The use of *β-casein* was (in retrospect) is not the best choice for a breast-specific promoter, not least because it was clearly expressed in non-breast tissues (Figure 4.4). Milk-protein genes such as *β-casein* are generally expressed during pregnancy and lactation in terminally differentiated cells, which die during mammary gland involution, and generally are not expressed strongly in ductal cells (both a site of normal *Brca1* expression and thought to be a common site of breast tumourigenesis (Marquis, 1995; Rijnkels and Rosen, 2001)). At the time the *c1* animals were generated, the *β-casein* mice were already in-house. A better choice would perhaps have been an epithelial-specific promoter (such as a keratin promoter), although these promoters are not solely breast-specific, or MMTV-Cre.

Several groups have suggested that cancer in general may have its genesis in mutated stem or progenitor cells, as by their nature they have long lifespans and undergo many cycles of replication (Medina, 2002; Smalley and Ashworth, 2003). The existence of mammary gland stem or progenitor cells is supported by a number of experiments investigating the ability of murine mammary epithelial cells to regenerate an entire mammary gland following transplantation into a cleared mammary fat pad (free of mammary epithelium following surgical separation of most of the fat pad from the nipple; this stops endogenous mammary epithelium from growing out and filling the fat pad with the mammary ductal tree structure). One group transplanted fragments of mammary epithelium from mice infected with MMTV into cleared fat pads to investigate if the reconstituted gland which grew out from the transplanted cells was a clonal population derived from a stem cell. They reasoned that if the reconstituted gland was clonal and the stem cell carried a proviral insertion, then the majority of the cells in the reconstituted gland would have the same proviral insertion. This was the case; Southern blot analysis indicated that some reconstituted glands appeared to be clonal, suggesting that a single stem cell progenitor might be able to give rise to an entire

mammary gland (Kordon and Smith, 1998). More recent work has shown that a subset of murine mammary epithelial cells efflux Hoechst 33342 dye, a property shared by haematopoetic and muscle stem cells (Goodell, 1996; Zhou, 2001; Alvi, 2003). Although this does not prove that mammary stem cells exist or that they are involved in tumourigenesis, another recent study has shown that only a subset of cells within a breast tumour (which can be segregated using cell-surface markers) are tumourigenic when injected into nude mice, indicating that perhaps only a limited number of cell types or lineages are involved in breast tumourigenesis (Al-Hajj, 2003).

Hormonally-regulated genes such as milk-protein genes are not likely to be expressed in stem or progenitor cell lines. Normally, use of a ubiquitously-expressed promoter might have the disadvantage of mis-expression of a transgene in tissues where it is not normally expressed. In this study, it was hoped that the ubiquitous expression of CMV-Cre might uncover a difference between the mouse and human *Brca1*-related tumour spectra by catalyzing loss of *Brca1* in a non–tissue-specific manner. Unfortunately, too few tumours have resulted from this study to allow conclusions about the tumour spectrum to be drawn.

4.3.2 Speculation on *gol/gol* mice

It is still unknown whether *gol/gol* mice will be viable. Based on the phenotype of the *gol/gol* ES cells, which appear to grow normally, it is postulated that homozygous mutant mice might be recovered, despite the fact that this allele is likely to give rise to a protein lacking the highly-conserved N-terminal RING domain.

However, another study has already indicated that full-length Brca1 protein may not be necessary for viability. Ludwig *et al.* have generated a mouse model which lacks the C-terminal half of the Brca1 protein - including the BRCT repeats – as a result of a truncating mutation in exon 11 (Table 1.3 #9) (Ludwig, 2001). This allele uncouples viability from loss of wildtype *Brca1*, although homozygous mutant mice are prone to tumourigenesis. A second

group has generated an allele which deletes only the second BRCT repeat (Table 1.3 #11), and mice homozygous for this mutation arrest by E10.5 (Hohenstein, 2001). The inviability of mice lacking only the BRCT repeat may be because of nonsense-mediated decay of the mutated transcript, although no publication has addressed such a possibility as yet. Therefore, while viability of mice homozygous for mutations in *Brca1* is not common, it does occur. A similar phenomenon is observed for the murine knockout alleles of a number of cancer-related genes, including *Brca2.* Donoho *et al.* generated a knockout allele of *Brca2* lacking exon 27, which codes for one of the Rad51 interaction domains. Mice homozygous for this mutation are recovered at the expected Mendelian ratio and are healthy and fertile, although they have an increased incidence of cancer and cells homozygous for this mutation are hypersensitive to DNA damaging agents (Donoho, 2003). In contrast, mice homozygous for other *Brca2* mutant alleles either exhibit early embryonic lethality, or, when live-born, are growth-retarded, generally infertile, and succumb to cancer at an early age (Connor, 1997; Ludwig, 1997; Sharan, 1997).

Ludwig *et al.* found that the viability of mice homozygous for the C-terminal truncation mutation depended partly on the strain background of the mice, an interesting finding which suggests that there are *Brca1* modifier loci which differ between mouse strains (Ludwig, 2001). If *gol/gol* mice do turn out to be viable, it will be very interesting to see if they show a similar modifier effect, and if they are fertile.

4.3.3 What the *gol* allele suggests about the *c1* allele

The viability of *gol/gol* ES cells begs the question of whether the excised *c1* conditional allele (*c1-*) is a null allele or not. *c1-* and *gol* differ only in the coding sequence of *Puro* and the lack of a PGK promoter to drive the *Puro* cassette (see Figure 3.3a and c). As the *c1-* allele was originally assumed to be a null allele, excision of the *c1* allele in *–/c1* ES cells in culture was not attempted; as described in Chapter 3, the primary concern was the efficiency of the *loxP* sites and the functionality of the *Puro* selection cassette. A

retrospective test of a *–/c1* ES cell line showed that it is possible to obtain *–/c1-* cells, as 2 of the 96 cell lines (2%) tested by Southern blot had undergone excision, suggesting that the *c1-* allele is a viable, non-null allele (data not shown). Based on this finding it is perhaps not surprising that mice carrying the conditional *c1* allele are not prone to tumourigenesis.

Although this may be an indication that, if viable, the *gol/gol* mice will also not be tumour-prone, mice entered into the *c1* tumourigenesis study were functionally wildtype, with mutation depending on Cre-mediated excision of the *c1* allele. This excision is a random event which could take place in any cell at any time, while should *gol/gol* mice be viable, they would already be homozygous for the mutation. The results from the *c1* tumourigenesis study may indicate that if *gol/gol* mice are viable, then it may be worthwhile to consider the tumour susceptibility of these mice in the presence of additional mutations, or following exposure to external mutagens such as γ-irradiation.

4.3.4 In conclusion

Although *gol/gol* mice have not yet been generated, the viability of *gol/gol* ES cells and inviability of *–/–* embryos and ES cells underscores the question of why these two alleles differ. The molecular differences between the *Brca1*$^{-}$ and *gol* alleles are discussed further in Chapter 6. Regardless of their differences, the existence of ES cells homozygous for the *gol* mutation affords an opportunity to study the consequences of the loss of the RING domain on the function of *Brca1*. It will be very interesting to determine if this domain is not required for viability of mice. In the following chapters, some of the phenotypes of *gol/gol* cells will be discussed.