CHARACTERIZATION OF A NOVEL DELETION ALLELE OF *Brca1*

by

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Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration, except where specifically indicated in the text. None of the material presented herein has been submitted previously for the purpose of obtaining another degree.

Debrah M. Thompson

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"Increase the Flash Gordon noise and put more science stuff around."
-Crow, MST3K, on how to make things look more "sciency."

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Mo beirt charide - tá sibh í mo chroi, agus do Éire, tugh tú dom síocháin.

Ach rinné mé an obair seo dhomh.

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ABSTRACT

Characterization of a novel deletion allele of *Brca1*

BRCA1 is a breast and ovarian cancer predisposition gene involved in human familiar breast cancer. Although its functions are not fully understood, it appears to be involved in DNA damage repair and genome stability. It is only 58% identical to its mouse homologue *Brca1*, but has two highly-conserved domains; an N-terminal zinc-finger RING domain, and two BRCT repeats at the C-terminus.

In this study, two murine knockout alleles of *Brca1* were generated. Both are missing exon 2, which contains the translational start site. The first (*Brca1*^{Brdm1}, referred to as "*Brca1*$^{-}$"), has the characteristics of previously described null alleles: *Brca1*$^{+/-}$ mice are healthy and not predisposed to tumourigenesis, and neither *Brca1*$^{-/-}$ embryonic stem (ES) cells nor mice could be generated. The second allele also replaces exon 2, but this allele (*Brca1*^{Brdm2}, named *gollum* and abbreviated as *gol*) does not behave like a null allele: *gol/gol* ES cells are viable and grow normally. The phenotypic differences between these two alleles may be due to the amount of *Brca1* transcript produced by each allele – ~2 kb more genomic sequence from intron 2 is deleted in *gol* than in *Brca1*$^{-}$. This area is postulated to carry a transcriptional repressor. Additionally, the protein produced from the *gol* allele (Brca1^{gol}) may be more stable than wildtype Brca1. Brca1^{gol} is predicted to lack a significant portion of the highly-conserved N-terminal RING domain, a region known to be important for interactions with protein partners, including Bard1, a nuclear chaperone of Brca1. In this study, it was demonstrated that Brca1^{gol} appears to be able to localize to the nucleus and will form DNA damage-induced nuclear foci, but has a decreased ability to bind to Bard1.

ES cells carrying the *gol* allele were tested for their response to various types of DNA damage. *gol/gol* and *gol/–* ES cells were hypersensitive to γ-irradiation and mitomycin C treatments, which cause double-strand breaks,

but did not appear to be hypersensitive to the base-damaging agents such as ultraviolet light or hydrogen peroxide. This indicated that the cells were deficient in double-strand break repair, which has two main components: homologous recombination repair (HRR) and non-homologous end-joining (NHEJ). Further analysis revealed that *gol/gol* cells had both a slight decrease in HRR efficiency and an increase in NHEJ efficiency as assayed by gene targeting and random plasmid integration.

gol, a novel deletion allele of *Brca1*, is of interest not only because it ablates a highly-conserved domain of the protein without conferring the expected loss of viability, but also because it has a clear defect in DNA damage repair. It offers a unique opportunity to further study the functions of Brca1.

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ABBREVIATIONS

5’ RACE	Rapid Amplification of cDNA Ends (5’ version)
6TG	6-thioguanine
aa	amino acid
AAP	Abridged Anchor Primer
Ab	antibody
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride
AR	androgen receptor
AT	ataxia-telangiectasia
ATM	Ataxia telangiectasia mutated
ATR	ATM and RAD3-related
AUAP	Abridged Universal Amplification Primer
BAC	bacterial artificial chromosome
BACH	BRCA1-accociated C-terminal helicase
BAP1	BRCA1-associated protein 1 (human protein)
BARD1	BRCA1- associated RING domain partner 1 (human protein)
Bard1	Brca1- associated RING domain partner 1 (mouse protein)
BARD1	BRCA1- associated RING domain partner 1 (human gene)
Bard1	Brca1- associated RING domain partner 1 (mouse gene)
BASC	BRCA1-associated genome surveillance complex
BAX	*BCL-associated X*, human gene
BCIP/NBT	5-Bromo-4-Chloro-3-Indolyl phosphate/nitro blue tetrazolium
BER	base excision repair
BLM	Bloom’s syndrome, causative gene (human)
Blm	Bloom’s syndrome, causative gene (mouse)
bp	base pair
BRC-1	*C. elegans BRCA1* homologue (gene)
BRCA1	*Breast cancer 1* (human gene)
BRCA1	Breast cancer 1 (human protein)
Brca1	Breast cancer 1 (mouse protein)
Brca1	*Breast cancer 1* (mouse gene)
Brca1^{-} (or “–“)	*Brca1*^{Brdm1}, null allele with *Hprt* cassette
Brca1-addPGK-TV	Targeting vector which adds PGK to the *c1(Puro corrected*) allele
Brca1-cond1-TV	*Brca1* conditional targeting vector, generates *Brca1*^{Brdc1}
Brca1-cond2-TV	*Brca1* conditional targeting vector, generates *Brca1*^{Brdc2}
Brca1-fixPuro-TV	*Brca1* targeting vector which fixes the *Puro* cassette of *c1*
Brca1^{gol}	Brca1 protein coded by the *Brca1*^{Brdm2} or *gol* allele

Brca1-gollum-TV	*Brca1* targeting vector, generates *Brca1*^{Brdm2} or *gollum*
Brca1-Hprt-TV	*Brca1 Hprt* replacement vector, generates *Brca1*^{Brdm1}
Brca1-Neo-TV	*Brca1 Neo* replacement vector
BRCA2	*Breast cancer 2* (human gene)
Brca2	*Breast cancer 2* (mouse gene)
BRCT	BRCA1 C-terminal
BRD-1	*C. elegans BARD1* homologue
BSA	bovine serum albumin
C	cysteine
c1	*Brca1*^{Brdc1} conditional allele
c1(+neo)	*Brca1*^{Brdc1} conditional allele with *Neo* selection cassette
c2	*Brca1*^{Brdc2} conditional allele
cM	centiMorgans
CMV	cytomegalovirus
co	conditional allele (in general)
Cre	cyclization recombination
CS	Cockayne's Syndrome
CstF	Cleavage stimulation factor
CtIP	CtBP-interacting protein
CtBP	C-terminal binding protein
Da	Dalton
DIG	digoxigenin
DMEM	Dulbecco's Modified Eagle Medium
DNA-PKcs	DNA-protein kinase, catalytic subunit
DSB	double-stand break
DSBR	double-strand break repair
dsDNA	double-stranded DNA
E	embryonic day
E2F1	E2F-transcription factor 1
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ER	oestrogen receptor
ES	embryonic stem
FANC	Fanconi anaemia protein
FANCD2	Fanconi anaemia protein D2 (human)
FBS	foetal bovine serum
FIAU	1-(2'-deoxy-2'-fluoro-b-D-arabinofuranosyl)-5-iodouracil
FISH	Fluorescent *in situ* hybridization
FITC	fluorescein isothiocyanate
G418	geneticin

GADD45	*Growth arrest and DNA damage inducible gene 45* (human)
Gapd	*Glyceraldehyde-3-phosphate dehydrogenase* (mouse gene)
Gdf-9	*Growth and differentiation factor-9* (mouse gene)
GGR	global genomic repair
gol	*Brca1*^{Brdm2}, or *gollum*
GSP	gene-specific primer
GST	glutathione-S-transferase
H	histidine
H_2O_2	hydrogen peroxide
HAT	hypoxanthine/aminopterin/thymine
HCC1937	human tumour- derived cell line carrying mutated *BRCA1* and *p53* genes (among other mutations)
Hprt	*hypoxanthine phosphoribosyltransferase* (mouse gene)
HRR	homologous recombinational repair
HSV-*tk*	herpes-simplex virus type 1 *thymidine kinase* (gene)
HT	hypoxanthine/thymidine
HU	hydroxyurea
i	innosine
IP	immunoprecipitation
JAK	Janus kinase
JNK/SAPK	c-Jun N-terminal kinase/stress-activated protein kinase
kb	kilobase
kDa	kilodalton
LIF	leukocyte inhibitory factor
LOH	loss of heterozygosity
loxP	*locus of crossover (P1)*
LTR	long terminal repeat
M-10	cell culture medium containing 10% serum
M-15	cell culture medium containing 15% serum
M17S2 (*NBR1)*	*Membrane component, Chromosome 17, Surface marker 2*
MDM2	*Mouse Double-Minute homologue 2*, human gene
Mdm2	Mouse double-minute 2 (protein)
MEF	mouse embryonic fibroblast
Melk	*Maternal embryonic leucine zipper kinase* (mouse gene)
MMC	mitomycin C
MMS	methyl methanesulfonate
MMTV	mouse mammary tumour virus
MOPS	3-(N-morpholino)propanesulfonic acid
Nbr1	*Neighbour of Brca1 1* (mouse gene)
NBR1	*Neighbour of BRCA1 1* (human gene)

NBR2	*Neighbour of BRCA1 2* (human gene)
NBS1	Nijmegen breakage syndrome protein
Neo	*Neomycin phosphotransferase*; antibiotic-resistance gene
NER	nucleotide excision repair
NES	nuclear export signal
NHEJ	non-homologous end joining
NLS	nuclear localization sequence
p21	$p21^{Waf1/Cip1}$
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PGK	promoter from the mouse *Phosphoglyceride kinase* gene
PI3K	phosphatidylinositol 3-kinase
PR	progesterone receptor
puro	puromycin, antibiotic
Puro	*puromycin N-acetyltransferase*; antibiotic-resistance gene
PVDF	polyvinylidene fluoride
pVHL	von Hippel-Lindau protein
RB1	Retinoblastoma protein
RB1	Retinoblastoma gene
revPGK	reveresed PGK promoter
RING	Really Interesting New Gene
RIPA	radioimmunoprecipitation
RNA Pol II	RNA Polymerase II holoenzyme
RNAi	RNA interference
RT-PCR	reverse transcription-polymerase chain reaction
scid	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SET	wash buffer, 0.15M NaCl, 20 mM Tris pH7.8, 1mM EDTA
SKY	spectral karyotyping
ssDNA	single-stranded DNA
STAT	signal transducer and activator of transcription
SV40	simian virus 40
T antigen	tumour antigen
TBST	wash buffer for protein blots (Westerns)
TCR	transcription coupled repair
Tris	Tris-Cl, buffer
TSG	tumour suppressor gene
TV	targeting vector
UTR	untranslated region
UV	ultraviolet

V(D)J recombination	variable (diverse) joining recombination
WT	wildtype
xBARD1	*Xenopus* homologue of BARD1 (protein)
xBRCA1	*Xenopus* homologue of *BRCA1* (gene)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XP	Xeroderma Pigmentosum
YAC	yeast artificial chromosome
Zn	zinc
β-gal	*β-galactosidase*
β-geo	*β-galactosidase/Neomycin phosphotransferase* fusion gene
β-ME	β-mercaptoethanol
γ	gamma
ΔX.11	*Brca1* splice isoform lacking exon 11
ΔX.2	*Brca1* splice isoform lacking exon 2

CHAPTER ONE:

INTRODUCTION

1.1 MOLECULAR MECHANISMS OF CANCER

1.1.1 Oncogenes

Cancer results from genetic mutations which disrupt the balance of cellular regulation, and is characterized by uncontrolled growth of mutated cells. Principal insights into the molecular mechanisms involved in cancer began with the identification of genes known as oncogenes; activated when mutated or overexpressed, oncogenes cause uncontrolled growth of cells, or reactivation of quiescent cells. These mutations are dominant, and generally are acquired somatically.

Studies with animal tumour viruses, particularly retroviruses, were instrumental in the discovery of oncogenes. Tumourigenic retroviruses can transform cells by modifying the activity of endogenous genes in various ways: direct mutation through proviral insertion, activation of a gene by the promoter and enhancer carried by a viral long terminal repeat (LTR), or through a non-essential, cell-derived, and mutated oncogene carried by the virus. For example, in tumours arising in mice infected with mouse mammary tumour virus (MMTV), the provirus often has affected the mouse oncogene *Wnt-1* (Nusse, 1984; Nusse, 1991). On the other hand, a transforming retrovirus such as the Rous sarcoma virus carries an oncogene (in this case *v-SRC*) derived from a normal, non-transforming chicken gene (*c-SRC*), which became part of the viral genome at some point in the past (Stehelin, 1976; Rous, 1983).

DNA tumour viruses transform cells through the interaction of a viral gene product with the host cell. For instance, the polyomavirus simian virus 40 (SV40) transforms through the action of its large T antigen (tumour antigen) gene product, which interacts with endogenous, host-derived regulatory proteins such as the retinoblastoma protein (RB1) and p53 (Lane and Crawford, 1979). Studies of viral-induced tumourigenesis contributed to the

realization that misregulation of either endogenous or foreign genes can result in uncontrolled cell growth.

1.1.2 Tumour Suppressors

The existence of negative factors in human cancer was postulated before the discovery of oncogenes, but the principal lines of evidence demonstrating the existence of tumour suppressor genes (TSGs) emerged later. Studies showed that fusions of tumourigenic and non-tumourigenic cells resulted in a non-tumourigenic cell, and that suppression of tumourigenesis depended on the presence of certain chromosomes (Harris, 1969). Working from a very different perspective, Alfred Knudson developed the two-hit model of carcinogenesis while studying familial retinoblastoma. Using statistical methods, he predicted that two mutations or "hits" were required for tumourigenesis in this syndrome, suggesting that retinoblastoma resulted not from the presence of an oncogene, but from the loss of what is now known as a tumour-suppressor gene (Knudson, 1971). Thus, in familial retinoblastoma, the first hit is an inherited germline mutation of one TSG allele. Loss of the second allele – or loss of heterozygosity (LOH) – by somatic mutation or chromosomal rearrangement then leads to tumourigenesis (reviewed in Knudson, 2000). In the case of sporadic retinoblastoma, both mutations are somatically acquired. This two-hit model is now applied to tumour-suppressor–related carcinogenesis in general.

More recently, TSGs have been divided into two broad classes based on their mechanism of action: "gatekeepers", or genes which inhibit the growth of tumours or promote apoptosis, and "caretakers", which regulate cellular processes that repair genetic lesions and maintain the overall genetic integrity of each cell (Kinzler and Vogelstein, 1997). The role of caretakers in tumourigenesis is less direct than that of gatekeepers: loss of genetic integrity in a cell which has lost both copies of a caretaker gene leads to the mutation of additional genes, which leads to cancer. This mechanism has been used

to explain why the mutation of some tumour-suppressors is rate-limiting for tumourigenesis, while mutations in others are not (Kinzler and Vogelstein, 1997). In the case of retinoblastoma, the gene involved, *RB1*, acts as a gatekeeper gene; it is directly involved in cell-cycle progression, and mutation of the second allele of *RB1* is the rate-limiting step in carcinogenesis (Knudson, 1971). On the other hand, inherited mutations in caretaker tumour suppressors lead to cancer by predisposing to secondary mutations through genomic instability. These secondary mutations are the rate-limiting step in carcinogenesis. Regardless of which type of gene is mutated, most studies agree that a number of mutations are generally required for progression to tumourigenesis (Knudson, 2001).

Cancer is one of the leading causes of death in the Western world (Centers-for-Disease-Control, 2000; Cancer-Research-UK, 2001). While much has been done to advance prevention, diagnosis, and treatment of various forms of cancer, there is still much work to be done toward understanding the process of tumourigenesis at the molecular level and the functions played by the various genes implicated in familiar cancer syndromes. The use of model systems, especially the mouse, has been instrumental in our understanding of cancer to date.

1.1.3 Mice as Models for Cancer

Before the rediscovery of Mendel's laws in 1900, mice were not extensively used in biological research, though mouse fanciers collected and bred the animals. Inbred strains of mice (generated by performing at least twenty generations of brother-sister matings) were developed to allow scientific study of animals with genetically homogeneous backgrounds. During the generation of the first inbred strains, it was noticed that some strains showed a high incidence of certain cancers – for example, the 129 strain was prone to testicular cancer (Stevens, 1970). Studies involving mutagenic agents identified a variety of chemical and physical methods which could be used to

reliably induce or enhance tumourigenesis in these mouse strains, though little was known about the specific genetic targets of these agents. To move beyond these molecularly uncharacterized and random methods of mutagenesis, techniques to stably and precisely introduce specific mutations into the mouse genome were needed. Transgenic mouse and embryonic stem (ES) cell technologies have met this need.

1.1.3.1 Transgenic and embryonic stem cell technologies

Transgenesis, the stable introduction of an exogenous gene into the germline of an organism (usually by pronuclear injection), was first demonstrated in the 1980s (Gordon and Ruddle, 1981). The first transgenic mouse models of cancer overexpressed viral oncogenes. More recently, models have been generated in which the activity of a transgene can be controlled temporally and spatially. This has enabled the production of mouse models that more accurately reflect the cascade of genetic events that characterize human malignancies (for a recent review, see Thompson, 2004).

ES cell technology provides a mechanism for the study of endogenous gene function by precisely targeting mutations into a gene or region of choice (Bradley, 1992; Bradley, 1998). This technology arose from two lines of research conducted in the 1980s.

First, it was demonstrated that pluripotent cells, later named ES cells, could be isolated from day 3.5 embryos and cultured (Evans and Kaufman, 1981). When reintroduced into blastocyst-stage embryos and implanted into host pseudo-pregnant females, these cells could repopulate the embryo, including the germ cell lineage, resulting in chimæric mice (Bradley, 1984). In these experiments, the host blastocysts were derived from unpigmented albino mice and the pluripotent cells from pigmented black agouti mice, meaning that the resulting chimæras (and the percentage of chimærism) were easily distinguishable. When chimæras are mated to albino mice, germline transmission of genetic material from the ES cells can be assessed by coat

colour: resulting progeny consist of albino mice and black agouti mice, half of which carry the targeted mutation (Bradley, 1984; Ramirez-Solis, 1993).

Second, it was demonstrated that ES cells could be modified in culture before being reintroduced into embryos to generate chimæras (Robertson, 1986; Thomas, 1986). The introduction of mutations or genomic changes into ES cells is generally accomplished through homologous recombination of a transfected targeting vector and the endogenous target genomic region (although other methods, such as retroviral-mediated insertion to generate mutations, also exist). Two types of targeting vectors are used (Figure 1.1a) (reviewed in Hasty, 2000). Insertion vectors carry one region of homology, require one homologous crossover event, and result in genomic integration of the entire targeting vector and a duplication of the region of homology. Replacement vectors carry two regions of homology and require two homologous recombination events for integration. Successful targeting of these vectors results in a specific change – be it a deletion, insertion, or mutation – of a region of the genome. Often, a vector will add a selectable marker to the successfully targeted area, for selection of targeted cells in culture. To date, targeting vectors and ES cell technology have been used to generate mutations in over 3,000 mouse genes (BioMedNet, 2003). ES cell technology can be used to mimic virtually any change in the genome, from single base pair mutations (Hasty, 1991) to large chromosomal deletions and inversions (Ramirez-Solis, 1995; Zheng, 1999a; Zheng, 1999b), and to generate a mouse model carrying the change.

1.1.3.2 Conditional Mutations and the Cre-loxP system

The coupling of site-specific recombinase systems with ES cell targeting vector technology has further refined the art of generating mouse models (reviewed by Kwan, 2002). A conditional targeting vector is very similar to a standard replacement vector, except that instead of replacing a region of the gene of interest, recognition sites for site-specific recombinases are inserted around the region (Figure 1.1b). Recombinase is then used to catalyze the

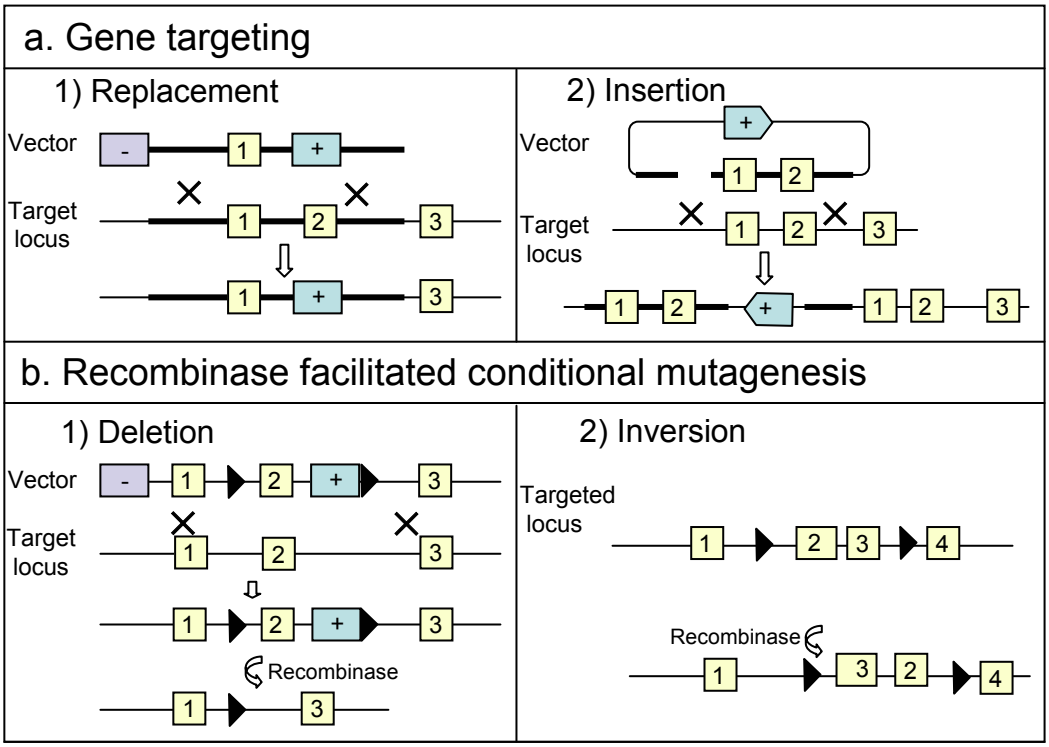


Figure 1.1: Gene-targeting vectors and recombinase-mediated mutagenesis. a. Replacement and insertion targeting vectors. **b.** Conditional targeting vectors, showing that deletion or inversion of the area will occur depending on the orientation of the recombinase recognition sites (black triangles). Figure taken from (Thompson, 2004).

deletion or inversion of the flanked area. The most commonly used recombinase system is the Cre/*loxP* system from bacteriophage P1. The *loxP* (*locus of crossover, P1*) recombinase recognition site is a 34 bp directional sequence recognized by the cyclization recombination (Cre) protein (Sauer and Henderson, 1988). The orientation of *loxP* sites is important; Cre-mediated recombination of two *loxP* sites flanking a region deletes the region if the *loxP* sites are in the same orientation, but inverts it if they are in opposite orientations. The Flp/frt recombination system, derived from the yeast *Saccharomyces cerevisiae,* has a similar mechanism of action, but is less widely used at present (Dymecki, 1996).

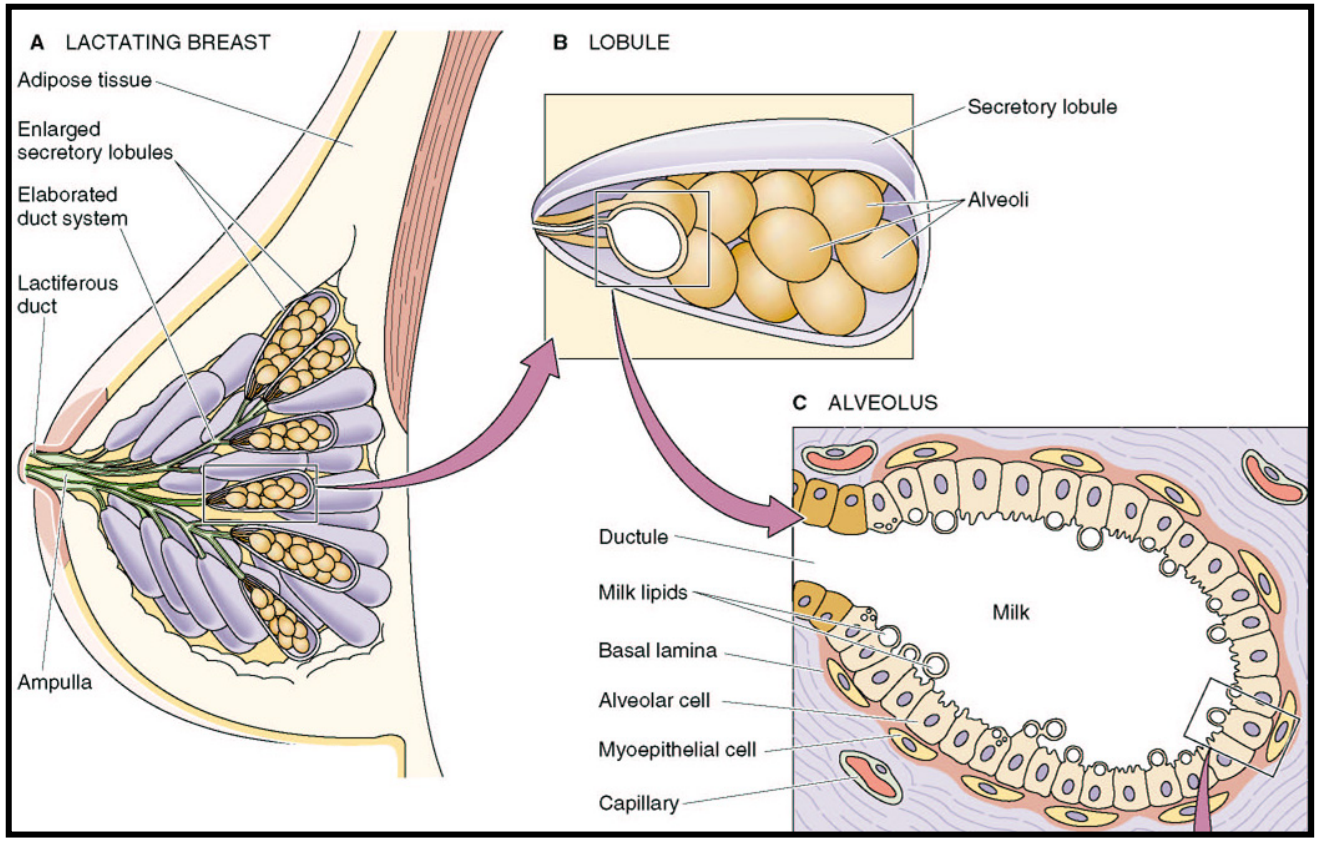
Such recombinase systems have proved to be very useful, both as an aid to circumventing embryonic lethality of a homozygous knockout and in generating more accurate models of human diseases. For example, when modeling human cancers, the optimal mouse model should reflect the biological, genetic, aetiological, and therapeutic aspects of the human cancer it models. High penetrance and short latency of tumourigenesis are desirable, because of the short mouse lifespan (Hann and Balmain, 2001). A common criticism of mouse models of human familial lesions is that the tumour spectra do not always mirror those of the human (Jacks, 1996), but some of the recently-reported conditional mutations are more faithful models of the human situation than are standard knockouts of the same genes (Shibata, 1997; Giovannini, 2000). The use of tissue-specific or adenoviral mechanisms of Cre delivery makes it possible to investigate the effects of a specific genetic lesion only in relevant tissues, or at a particular time. Furthermore, as recombinase efficiency is rarely 100%, conditional mutants provide a mechanism for modeling both the random nature of mutagenesis in human cancers and the microenvironment of a mutant cell amongst normal ones.

1.2 BREAST CANCER

1.2.1 Human breast cancer: a brief overview

One in nine women in the United Kingdom is predicted to develop breast cancer within her lifetime (Cancer-Research-UK, 2003). When considered in light of the impact that this disease has, not only on patients, but also on their family and friends, it is a very relevant topic of study and understandably the focus of much research. The mammary gland has a unique physiology in that it may undergo several rounds of growth, terminal differentiation, and regression during development and multiple pregnancies. The primary breast architecture is laid down during development, and further development occurs during puberty. Hormonal signals during pregnancy trigger large amounts of growth and differentiation to facilitate milk production; cessation of breast-feeding at weaning signals the regression of much of this growth (Figure 1.2d). The breast itself consists of around twenty lobes, each of which has a branching structure of ducts leading to ductules leading to lobules. The lobules contain the alveoli (or acini), where milk-producing cells are located. Epidermal cells lining the alveoli are hormonally stimulated to produce and secrete milk proteins. Milk then travels down the duct system to the nipple (Figure 1.2).

The two major forms of breast carcinoma are classified as ductal and lobular carcinomas, although many texts suggest that the majority of breast carcinomas arise from the terminal ductule-lobule units, regardless of their classification (Aldaz, 2002; Bulpaep, 2003). Others suggest that cancer arises from either mammary stem cells or ductal progenitor cells, as the majority of resulting cancers are ductal in nature (Medina, 2002). This stem cell theory may be supported by a recent finding 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 (Al-Hajj, 2003).



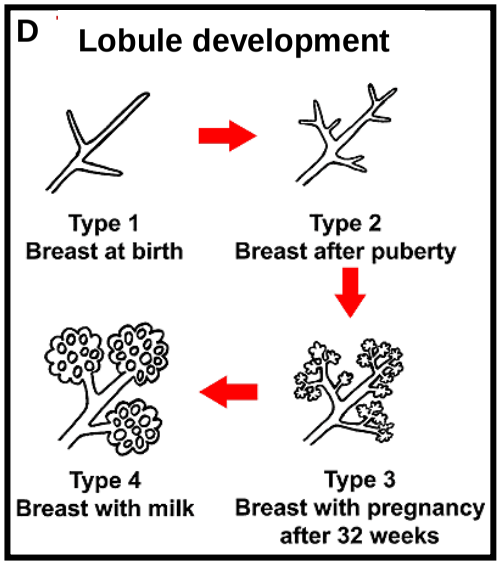


Figure 1.2: Architecture of the human breast, milk production, and breast development.
a. Schematic showing the branching duct-to-lobule structure of the breast during lactation. **b.** The lobule contains the alveoli (also called acini), where the milk-secreting cells are located. **c.** The epidermal cells lining the alveoli secrete the milk. **d.** Development of branching duct-lobule structure; pregnancy triggers the branching and differentiation of ducts and lobules in preparation for milk production.
Figures taken from (Bulpaep, 2003; Brind, 2002).

1.2.2 Hereditary breast cancer and the familial predisposition gene *BRCA1*

That breast cancer might have a hereditary component became clear as early as the mid-nineteenth century. While mutations in familial predisposition genes account for less than 10% of all human breast cancer cases, an estimated 40% of early-onset (before the age of 30) cases are attributable to such mutations (Claus, 1991). The normal lifetime risk of breast cancer by the age of 70 for women in the UK is ~6.7%, but carriers of a mutated predisposition gene have a increased lifetime risk of breast cancer of ~65% (estimates range from 35.3% to 70%) by age 70 (Easton, 1993a; Antoniou, 2002; Antoniou, 2003; Cancer-Research-UK, 2003; King, 2003). A large-scale study undertaken by the Centers for Disease Control suggested an autosomal dominant form of inheritance for familial breast cancers (Claus, 1991).

In 1990, Mary-Claire King's group at the University of California at Berkeley reported a linkage between a region on human chromosome 17q21 with the causal mutation carried by a number of breast-cancer families in which early-onset breast cancer was common (Hall, 1990). In the four years between the appearance of this paper and the successful cloning of the gene known as *BRCA1* (*Breast Cancer 1*), a host of studies were published which provide a picture of positional cloning methods in the pre-genome–sequence era. The region on 17q was the target for the development of radiation hybrid maps (Abel, 1993; Black, 1993) and the assembly of high densities of markers for analysis (Anderson, 1993). Yeast artificial chromosome (YAC) and P1 contigs were used for physical mapping and identification of candidate genes (Albertsen, 1994). Numerous groups reported collections of breast-cancer families linked to the slowly narrowing target region (Devilee, 1993; Easton, 1993b; Spurr, 1993). In 1994, *BRCA1* was identified and its sequence published (Miki, 1994). Confirmation from other groups that this newly identified gene was indeed mutated in families with breast or breast and ovarian cancers swiftly followed (Friedman, 1994; Futreal, 1994). Evidence that *BRCA1* is a tumour suppressor gene emerged even before it was cloned.

In a study of breast and ovarian tumours from families whose disease was linked to chromosome 17q, a majority had lost heterozygosity of the wildtype chromosome in the region where *BRCA1* was believed to be located (the second hit, according to the Knudson hypothesis (Knudson, 1971)) (Smith, 1992).

BRCA1 spans approximately 100 kilobases (kb) of genomic sequence, and is composed of 23 exons which encode a 7.8 kb mRNA. Exon 1 is non-coding, and the region originally identified as exon 4 is an Alu repeat not generally included in the transcript (Miki, 1994). The 220 kiloDalton (kDa) protein shows a predominantly nuclear localization, and forms nuclear "dots," or foci, during S phase of the cell cycle and following DNA damage (Chen, 1995; Scully, 1996; Scully, 1997b). Although initial characterization indicated that the protein had no significant homology to any other sequences in the databases, it does have two features: an N-terminal zinc (Zn)-finger domain, known as a RING-finger, and a C-terminal region with two tandem repeats of a small domain known as a BRCA1 C-terminal, or BRCT, motif (Koonin, 1996). BRCT motifs are also found in other proteins involved in DNA repair or cell-cycle control such as p53 binding-protein 1, XRCC1, and RAD9 (Koonin, 1996; Bork, 1997; Callebaut and Mornon, 1997).

1.2.3 Other familial breast-cancer predisposition genes

While the hunt for *BRCA1* was ongoing, one group noticed that the susceptibility gene in some of the breast-cancer families they analyzed mapped to a different region. This region, located on chromosome 13q, was proposed to harbour a second predisposition gene, *BRCA2* (*Breast Cancer 2*) (Wooster, 1994). Positional cloning techniques were used initially to locate the *BRCA2* gene, but *BRCA2* was ultimately identified using newly-released sequence data from the Human Genome Project. Only fifteen months after publishing the original linkage paper, the same group confirmed that mutations in *BRCA2* were indeed present in the breast-cancer families linked to chromosome 13q (Wooster, 1995). Like BRCA1, the BRCA2 protein was

not similar to other proteins in the databases and had neither a RING domain nor BRCT repeats (Tavtigian, 1996).

More recently, a mutation in a third gene, *CHEK2*, was linked with a small number of familial breast cancer cases (Meijers-Heijboer, 2002; Vahteristo, 2002). Other genes linked to a higher incidence of breast cancer include *p53* and *ATM*, the gene mutated in the human disorder ataxia-telangiectasia. It is likely that other predisposition genes exist, but are either rare or show low penetrance (Antoniou, 2002; Wooster and Weber, 2003).

1.3 CHARACTERISTICS OF *BRCA1*-RELATED HUMAN TUMOURS

1.3.1 Cancer-related *BRCA1* mutations and the risk they confer

According to two recent analyses of *BRCA1*-related breast-cancer families, *BRCA1* mutation carriers have a ~65% overall risk of breast cancer by the age of 70, a 14- to 30-fold increased risk of breast cancer (depending on age) relative to that of non-carriers (Antoniou, 2003; King, 2003). *BRCA1* mutations also confer an increased risk of other cancers, most notably ovarian cancer (~40% overall risk by the age of 70). This will be discussed further in section 1.3.3.

Cancer-related *BRCA1* mutations include small insertions or deletions which cause frameshifts, and nonsense or missense mutations; premature truncation of the mutant protein is common (Friedman, 1994; Gayther, 1995). For a detailed and up-to-date listing of *BRCA1* mutations, see the Breast Cancer Information Core website (BIC, 2003)). Generally, *BRCA1* does not have mutation “hotspots” (unlike the TSG *p53* (Walker, 1999)), although some mutations are more commonly observed, such as the missense mutation C61G, a mutation in the highly-conserved RING domain, or the frameshift mutations 186delAG (in exon 2) and 5382insC, located in one of the BRCT repeats (BIC, 2003). Intronic or exonic mutations which change the normal splice pattern have also been identified (Gayther, 1995; Xu, 1997b). Cancer-related mutations occur all throughout *BRCA1*, from the 5’ end down to a

mutation which gives rise to a protein lacking only the last few amino acids (BIC, 2003). While there does not appear to be a bias in mutation position in regards to tumourigenesis in general, the frequency of ovarian cancers relative to breast cancers is higher in families carrying mutations in the middle of the gene (mostly in exon 11) than at either of the ends (Gayther, 1995; Thompson and Easton, 2002).

1.3.2 *BRCA1* and sporadic breast and ovarian cancers

A number of studies have shown that BRCA1 protein and/or mRNA appears to be down-regulated in some sporadic breast cancers, regardless of their stage of progression (Thompson, 1995; Magdinier, 1998; Rio, 1999; Baldassarre, 2003). The exact cause of this loss of expression is unknown. A percentage of tumours with decreased *BRCA1* expression have undergone LOH at the *BRCA1* locus, but others have not – and some samples with LOH express *BRCA1* at normal levels (Thompson, 1995; Sourvinos and Spandidos, 1998; Rio, 1999; Staff, 2003). In general, LOH at the *BRCA1* locus in sporadic breast cancers does not appear to be critical for tumourigenesis, which is not surprising, as LOH of *BRCA1* in sporadic tumours would not be expected to have a detrimental effect unless one allele was previously modified or mutated (Futreal, 1994; Merajver, 1995). While promoter hypermethylation could provide such a modification, only a small subset of the sporadic breast cancers studied to date show aberrant methylation of the *BRCA1* promoter region (Magdinier, 1998; Rice, 1998). In familial breast cancers, a small study has demonstrated that promoter methylation rarely serves as the second *BRCA1* hit, with LOH the more common mechanism (Esteller, 2001). Although loss of *BRCA1* expression may contribute to sporadic breast cancer, the mechanism behind this downregulation, or the roles it plays in tumourigenesis, have yet to be determined.

1.3.3 *BRCA1*-related, *BRCA2*-related, and sporadic breast cancers

The phenotypic consequences of a *BRCA1* mutation differ from those of a corresponding *BRCA2* mutation (Tables 1.1 and 1.2). The overall risk of breast cancer for carriers of *BRCA2* mutations has been estimated at 45–74% by the age of 70, but their risk for ovarian cancer (~11% overall risk by the age of 70) is lower than that of carriers of *BRCA1* mutations (Antoniou, 2003; King, 2003). Male carriers of *BRCA2* mutations have a ~7% overall risk of breast cancer by the age of 80; mutations in *BRCA2* are predicted to account for approximately 10% of all male breast cancers (Thompson and Easton, 2001). Male breast cancer is not common in *BRCA1* families (Antoniou, 2003). Small increased risks for other cancers have been reported for both genes (Table 1.1) (Consortium, 1999; Brose, 2002).

BRCA1-related human breast tumours are also pathologically distinct from both *BRCA2*-related or sporadic breast tumours (Lakhani, 1998; Armes, 1999). Table 1.2 reports some of the differences between *BRCA1*- or *BRCA2*-related and sporadic breast tumours, including common secondary mutations, responsiveness to hormones, and *p53* mutation status. *p53* mutations are observed more frequently and tend to occur at less commonly-mutated sites in *BRCA1* and *BRCA2* breast tumours than in sporadic breast tumours (Ramus, 1999; Greenblatt, 2001). *BRCA1*-related ovarian cancers are also more likely to carry a mutation in *p53*, although the overall *p53* mutation spectrum in these tumours is very similar to that of sporadic ovarian cancers (Buller, 2001). Additionally, while the pathology of *BRCA1*-related and sporadic breast tumours differ, the pathology of *BRCA1*-related and sporadic ovarian cancers is very similar (Rubin, 1996).

1.4 *Brca1* – the mouse homologue of *BRCA1*

The murine homologue of *BRCA1* (*Brca1*) has 23 coding exons which encode a ~7.2 kb mRNA and a protein of 1812 amino acids (aa) (Abel, 1995; Bennett, 1995; Lane, 1995; Sharan, 1995; Schrock, 1996a). Its location on mouse chromosome 11 correlates with earlier studies which revealed a large linkage

Table 1.1: Cancer risks for carriers of deleterious mutations in *BRCA1* or *BRCA2.*

Characteristic	*BRCA1*	*BRCA2*	References
Risk of breast cancer (by age 70)	~65%	45%[$]	(Johannsson, 1997; Armes, 1999; Lakhani, 2002; King, 2003)
Risk of ovarian cancer (by age 70)	~40%	~11%	(Johannsson, 1997; Armes, 1999; Lakhani, 2002; King, 2003)
Risk of male breast cancer (by age 80)	Not generally observed	7%	(Ramus, 1999; Greenblatt, 2001)
Increased risk of other cancers[*]	Colon, pancreatic, gastric, fallopian tube (female)	Pancreatic, gall bladder, stomach, malignant melanoma, prostate (male)	(Consortium, 1999; Brose, 2002; Thompson and Easton, 2002; Edwards, 2003)

* Observed incidence of other cancers was low; generally the risk relative to that of non-carriers was 2- to 4-fold higher.
[$] Estimated at 74% in (King, 2003).

Table 1.2: *BRCA1* and *BRCA2*–related breast tumours: characteristics compared to sporadic breast tumours.

Characteristic	*BRCA1*	*BRCA2*	Sporadic	References
Loss of oestrogen receptor (ER) activity	90%	34%	35%	(Johannsson, 1997; Armes, 1999; Lakhani, 2002)
Loss of progesterone receptor (PR) activity	79% (or higher)	45%	41%	(Johannsson, 1997; Armes, 1999; Lakhani, 2002)
p53 mutations	More common			(Ramus, 1999; Greenblatt, 2001)
p53 mutated at "hotspots *"	67%		82%	(Greenblatt, 2001)
Multiple *p53* mutations	16%		4%	(Greenblatt, 2001)
Loss of *ErbB2/neu/Her-2* expression	97%	97%	85%	(Lakhani, 2002), supported by (Johannsson, 1997; Armes, 1999)
Loss of Cyclin D expression	Lost in 6 of 9 cases (small study)	Lost in 5 or 9 cases (small study)	Retained in 19 control cases (small study)	(Armes, 1999)
Amount of LOH	Increased			(Tirkkonen, 1997)
LOH hotspots	5q, 4q		8p, 16q	(Tirkkonen, 1997)
Aneuploidy	More common			(Johannsson, 1997)

*p53 codons frequently mutated in human cancers (Walker, 1999).
LOH=loss of heterozygosity.

group of over 50 centiMorgans (cM) shared between human chromosome 17, mouse chromosome 11, and rat chromosome 10 (Remmers, 1992; Yamada, 1994). Overall, the BRCA1 mouse-human protein identity is 57%, but there are two regions of very high homology: the N-terminal region (97% similar; within the RING motif, the identity is 100%) and the C-terminal BRCT repeats (83% identical), which underscores the importance of these two domains (Figure 1.3) (Sharan, 1995). Other mammalian *BRCA1* homologues from the rat (Bennett, 1999) and dog (Szabo, 1996) also demonstrate this low overall conservation but high conservation at the two terminal domains (Szabo, 1996).

One feature of *BRCA1* which differs between the mouse and human homologues is the 5' untranslated region (5' UTR). While both genes are TATA-less (Rice, 1998) and appear to share a bidirectional promoter with another, head-to-head oriented gene, the human *BRCA1* 5' region is more complex (Figure 1.4). Mouse *Brca1* lies head-to-head with a gene called *Nbr1* (*Neighbour of Brca1 1*) (Chambers and Solomon, 1996; Dimitrov, 2001), but the human 5' region contains, in addition to a head-to-head copy of *NBR1* (*Neighbour of BRCA1 1*, also known as *M17S2*, *Membrane component, Chromosome 17, Surface marker 2*) (Campbell, 1994), two differentially spliced copies of exon 1 of *BRCA1* (Xu, 1995), a second gene called *NBR2* (*Neighbour of BRCA1 2*) (Xu, 1997a), and a few *BRCA1* pseudo-exons. This additional complexity likely results from a partial duplication of the region (Brown, 1996). The rat 5'UTR is very similar to that of the mouse (Bennett, 1999).

1.5 FUNCTIONAL CHARACTERISTICS OF *BRCA1*

1.5.1 Tissue expression profile of *BRCA1* in mice and humans

BRCA1 is expressed in many human tissues, including breast and ovary, thymus, kidney, and testis (Miki, 1994). In human testes, both *BRCA1* and *BRCA2* are highly expressed in zygotene and pachytene spermatocytes (Scully, 1997c; Chen, 1998). Murine *Brca1* transcript is also detected in testis

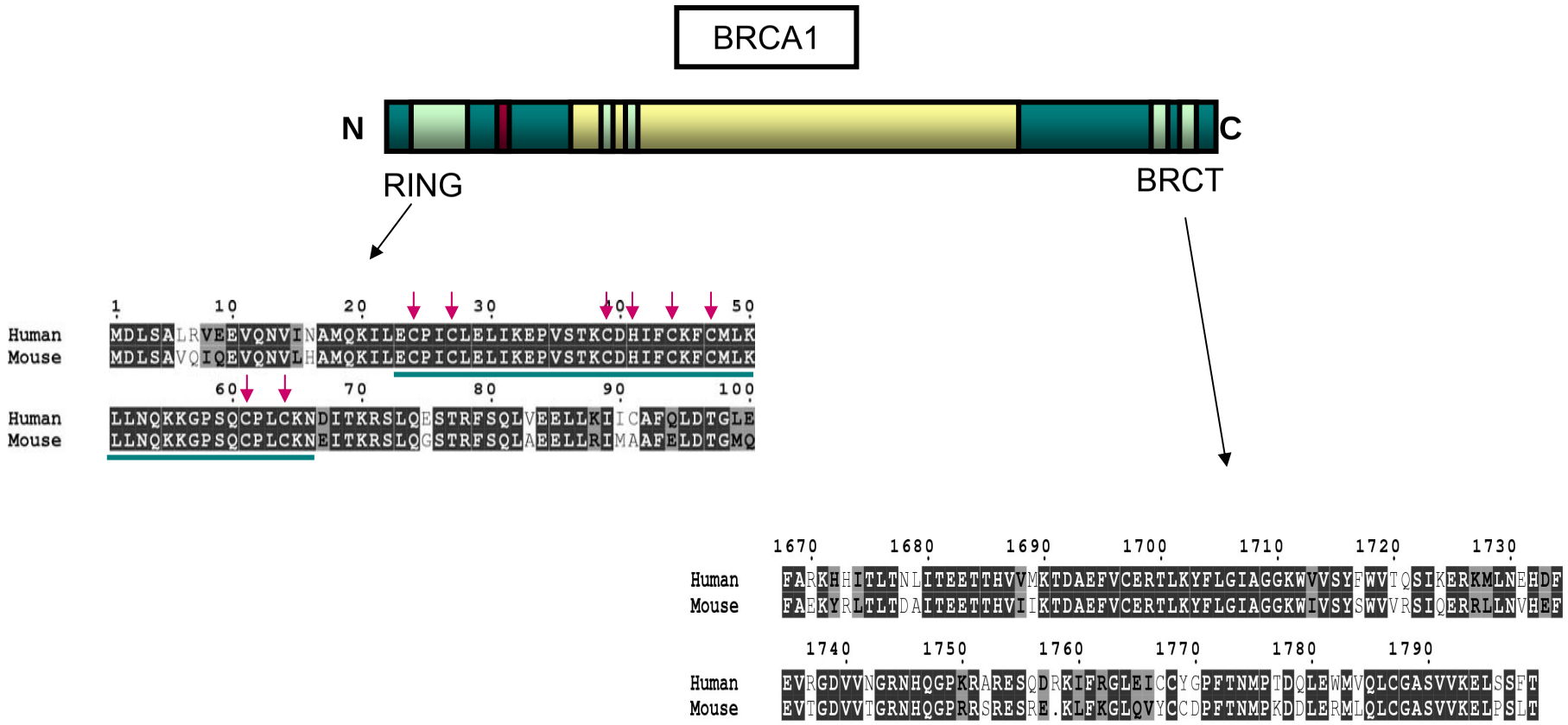


Figure 1.3: Conservation of mouse and human BRCA1 proteins. Schematic of BRCA1 showing the conserved RING domain and BRCT repeats. The yellow box represents exon 11, the largest coding exon. Boxes show the human-mouse protein alignments for the indicated regions. For the RING domain, the key cysteine (C) and histidine (H) residues are indicated by red arrows and the canonical RING motif is underlined in green. Identical residues are highlighted in dark grey, similar ones in light grey. ClustalW and ESPript (Blosum62 matrix) were used to produce the alignments.

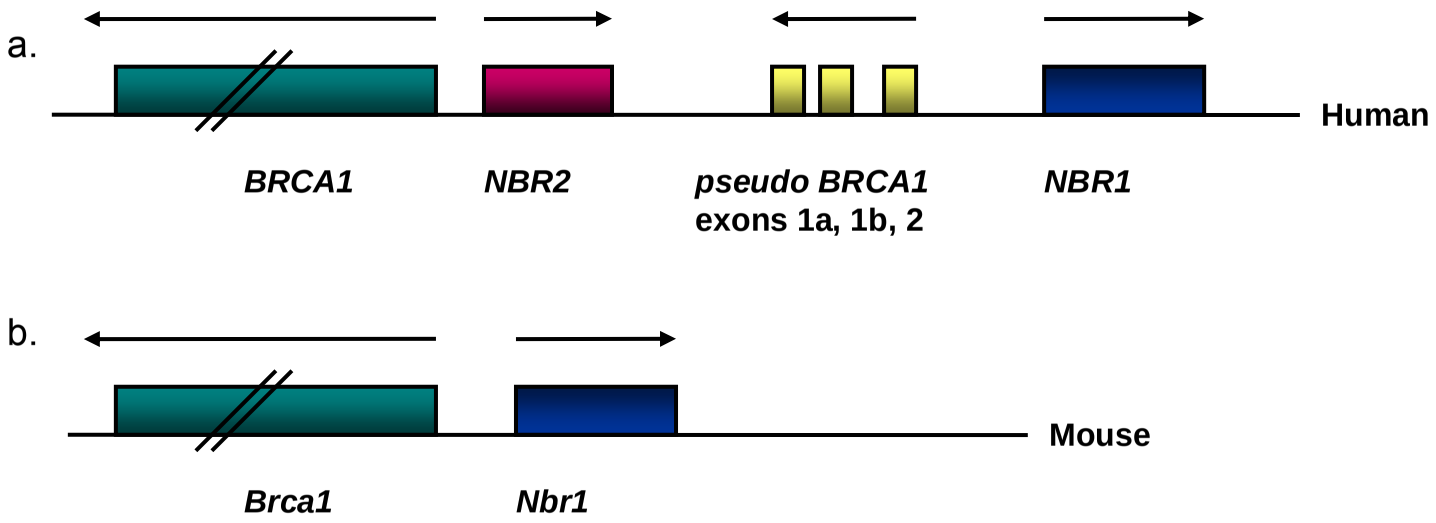


Figure 1.4: Organization of the genomic region upstream of mouse and human *BRCA1*.
a. The genomic region upstream of human *BRCA1* contains two other genes and three *BRCA1* pseudo-exons. **b**. The murine upstream genomic region is less complex. Information derived from mouse and human Ensembl and genome sequences. Arrows indicate the direction of transcription of each gene. The *NBR* genes do not share sequence homology.
Abbreviations: *NBR2*=human *Neighbour of BRCA1 2* gene, *NBR1*=human *Neighbour of BRCA1 1* gene, *Nbr1*=mouse *Neighbour of Brca1 1* gene.

(Lane, 1995; Marquis, 1995). Consistent with the human expression profile, *Brca1* is highly expressed in pachytene spermatocytes and round spermatids, and expression of the transcript increases as the testes develop (Zabludoff, 1996), suggesting that BRCA1 plays a role in meiosis. Supportingly, male infertility has been observed in *Brca1* mice homozygous for either an allele lacking all of exon 11 or one which carries a truncated protein. In both models, spermatogenesis arrested during the prophase stage of meiosis I, although one mutant was only examined in the context of a $p53^{+/-}$ or $p53^{-/-}$ background (Cressman, 1999a; Ludwig, 2001; Xu, 2003).

In the mouse, *Brca1* is widely expressed during development, but in adult animals it appears to be expressed mainly in proliferating cell types involved in differentiation (Marquis, 1995). *Brca1* is expressed in the epithelial cells of the breast, and its expression is increased during pregnancy and lactation, especially in the rapidly growing and differentiating terminal end buds and alveoli. Increased expression of *Brca1* in breast tissue can be induced by oestrogens and progesterone, although this induction may be an indirect effect, as *Brca1* appears to be expressed in growing cells and hormone signaling results in increased proliferation of breast tissue (Lane, 1995; Marquis, 1995). This supposition is supported by *in vitro* studies using human oestrogen-responsive breast cancer cell lines, which demonstrate that upregulation of *BRCA1* expression is delayed by nearly 24 hours following oestrogen stimulation, suggesting that oestrogen does not directly upregulate *BRCA1* (Gudas, 1995; Spillman and Bowcock, 1996; Marks, 1997). The role of BRCA1 in differentiation has been suggested by *in vitro* studies, as a mammary epithelial call line can be induced to differentiate by ectopically expressing *BRCA1* in conjunction with hormonal triggers (Kubista, 2002).

1.5.2 Expression of alternative forms of *BRCA1*

Screening of *BRCA1*-related breast tumours has identified several splice aberrations which appear to be associated with tumourigenesis (Xu, 1997b). Analyses of *BRCA1* transcripts in normal cells has demonstrated that *BRCA1* may normally be expressed in more than one form. Two alternative forms of

BRCA1 have been described which lack all (∆*X.11*) or most (∆*X.11b*) of exon 11, the largest exon (Thakur, 1997; Wilson, 1997). Both of these isoforms were identified by reverse transcription of human cellular RNA followed by amplification by the polymerase chain reaction (RT-PCR) using *BRCA1*-specific primers. The mouse has a similar (single) natural ∆*X.11* splice isoform, and this conservation suggests that the full-length and ∆*X.11* forms of *BRCA1* may both be biologically relevant, perhaps with different roles in the cell (Xu, 1999c; Bachelier, 2000).

These ∆*X.11* splice isoforms will be discussed throughout this chapter, as they have proved useful in studying the functions of BRCA1. When expressed at physiological levels, BRCA1 ∆X.11 proteins form S phase and damage-induced nuclear foci as does full-length BRCA1 (Xu, 1999c), and are recognized by BRCA1 antibodies raised to the N- or C-terminal ends of the protein. However, a glance at Figure 1.5 (exon 11 is depicted in yellow) shows that exon 11 codes for the part of the BRCA1 protein thought to be important for interaction with proteins such as RAD51 and BRCA2, and thus this isoform will likely not participate in RAD51/BRCA2-related functions, thought to be important for DNA repair. This supposition is supported by experiments using mouse embryonic fibroblasts (MEFs) generated from mice expressing only the *Brca1* ∆*X.11* isoform. These cell lines have a defective G2-M checkpoint and are more likely to have extra centrosomes than wildtype MEFs (Xu, 1999c). Furthermore, ectopic expression of *BRCA1* ∆*X.11* in a mammary epithelial cell line will not induce differentiation while full-length *BRCA1* will (Kubista, 2002).

Besides the ∆*X.11* splice isoforms, human *BRCA1* also has two alternative first exons, exon 1a and 1b (Xu, 1995). Other reports suggest that a third first exon may exist, a truncated form of 1a called 1a' (Hsu, 2001; Jakubowska, 2001). Murine *Brca1* appears to have only one exon 1. In both human and mouse, exon 2 contains the translational start site, so the alternative use of first exons in human cells is postulated to have some regulatory role. It has further been proposed that human *BRCA1* is expressed from two alternative

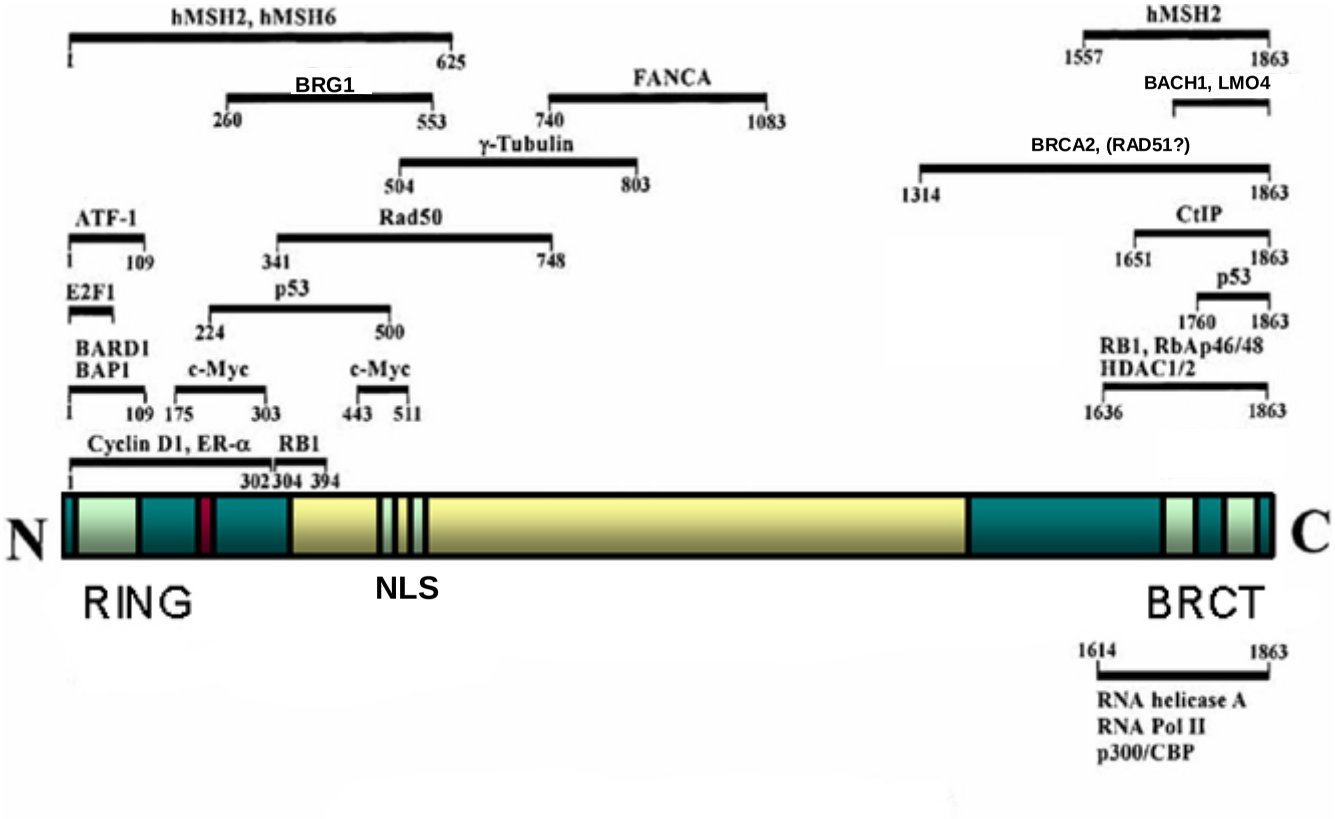


Figure 1.5: BRCA1 interacts with many other proteins at various sites. Schematic showing where BRCA1 is thought to interact with other proteins. This list is not comprehensive. The part of the protein coded by exon 11 is depicted in yellow.
Abbreviations: ATF-1=activating transcription factor-1, BACH1=BRCA1-Associated C-terminal Helicase 1, BAP1= BRCA1-associated protein 1, BARD1=BRCA1-associated RING domain protein-1, BRCT=BRCA1 C-terminal repeat, BRG1= *Brahma*-related gene 1, CBP=p265 CREB binding protein, CtIP=CtBP (C-terminal binding protein)-interacting protein, ER-α= oestrogen receptor-α, FANCA=Fanconi anaemia protein A, HDAC1/2=histone deacetylase-1/2, LMO4=LIM domain-only 4, hMSH2, hMSH6=MutS homologues 2 and 6, NLS=nuclear localization signal, RB1=Retinoblastoma protein, RbAp46/48= retinoblastoma-associated protein 46/48 kDa, RNA Pol II=RNA Polymerase II, RING=zinc finger RING domain.
Adapted from (Rosen, 2003).

promoters (Xu, 1997c), but this finding has been contested by another group (Suen and Goss, 2001).

1.5.3 BRCA1 and the cell cycle

BRCA1 protein and mRNA levels are dynamic. While *BRCA1* appears to be ubiquitously expressed in growing cells (Marquis, 1995; Chen, 1996; Ruffner and Verma, 1997), maximum expression levels occur at the G1-S boundary and during S phase (Gudas, 1996). In a similar manner, BRCA1 protein levels are highest during S phase. BRCA1 protein is also phosphorylated at different levels; hyperphosphorylation occurs during the G1-S transition, and the modification remains throughout M phase; partial dephosphorylation occurs in early G1 (Ruffner and Verma, 1997; Scully, 1997b). BRCA1 protein is mainly nuclear, with a diffuse staining pattern, but is often observed in S phase nuclear "foci" which persist until G2 (Scully, 1996; Scully, 1997c). The biological relevance of these foci is still unknown. Following DNA damage, there is at least a transient upregulation of BRCA1 protein and mRNA levels, and the protein becomes hyperphosphorylated (more so than in S phase) and localizes to damage-induced nuclear foci (Scully, 1997b; MacLachlan, 2000a).

The relationship of BRCA1 levels to cell cycle phases led to the suggestion that BRCA1 might be involved in the G1-S and/or G2-M cell-cycle checkpoints, a supposition now supported by several lines of evidence. There have been reports that BRCA1 may be involved in the S phase transition, as well; one group studying the HCC1937 cancer cell line reported a defective S phase checkpoint in these cells following gamma (γ)-irradiation (Xu, 2001a). Restoration of the defective checkpoint occurred upon transient expression of *BRCA1* (Xu, 2001a; Xu, 2002). However, another group studying the HCC1937 cell line reported a normal S phase checkpoint following γ-irradiation (Scully, 1999), suggesting that further study is needed to determine the role of BRCA1 in this checkpoint. The evidence linking BRCA1 to the G1-S and G2-M checkpoints is more compelling.

1.5.3.1 BRCA1 and the G1-S checkpoint

The G1-S cell-cycle checkpoint exists to prevent the replication of damaged DNA. Progression through this checkpoint requires the kinase/cyclin pairs CDK4/Cyclin D and CDK2/Cyclin E. The CDK2 and CDK4 kinases regulate proteins involved in S phase promotion, including inactivating the suppressor protein RB1 and activating CDC45. CDK2/Cyclin E appears to be a main target for the damage-induced G1-S checkpoint; following damage, the kinases ATM and ATR (through CHK1 and CHK2) destroy the phosphatase CDC25A, which normally activates cyclin D and cyclin E (this a rapid response through to result from proteasome-mediated degradation). Additionally, the p53 protein is stabilized (reviewed in Iliakis, 2003). p53 stabilization has various effects, including upregulation of the cell-cycle related gene $p21^{Waf1/Cip1}$ (referred to here as *p21*). As p21 is a potent suppressor of CDK2, stabilization of p53 is a second pathway for blocking progression into S phase (el-Deiry, 1993; Harper, 1993).

Overexpression of *BRCA1* has been shown to result following DNA damage (Clarkin, 2000; MacLachlan, 2000a). Overexpression of *BRCA1* in cultured cells has also been shown to cause growth suppression in conjunction with an arrest in the G1 phase of the cell cycle (Somasundaram, 1997; Aprelikova, 1999), as well as slowed development of MCF-7 (a human breast cancer cell line)–derived tumours in nude mice (Holt, 1996). However, overexpression of *BRCA1* does not suppress the growth of cells lacking either RB1 or p21 (Somasundaram, 1997; Aprelikova, 1999). Since BRCA1 can upregulate *p21* (Somasundaram, 1997), this suggests that overexpression of *BRCA1* inhibits S phase progression through its effect on p21, which normally suppresses the activity of CDK2. If this is the case, cells lacking p21 or RB1 (which is normally activated by CDK2 to stimulate progression into S phase) would then be expected to be insensitive to *BRCA1* expression levels (see Figure 1.6 for a simplified diagram) (Zhang, 1998; Aprelikova, 1999; MacLachlan, 2000b). Overexpression of *BRCA1* may also aid in stabilization of the p53 protein; two different regions of BRCA1 appear to interact with p53 (see Figure 1.5) (Somasundaram, 1999). Either stabilization of p53 or upregulation of *p21* should result in a G1-S block, as discussed above.

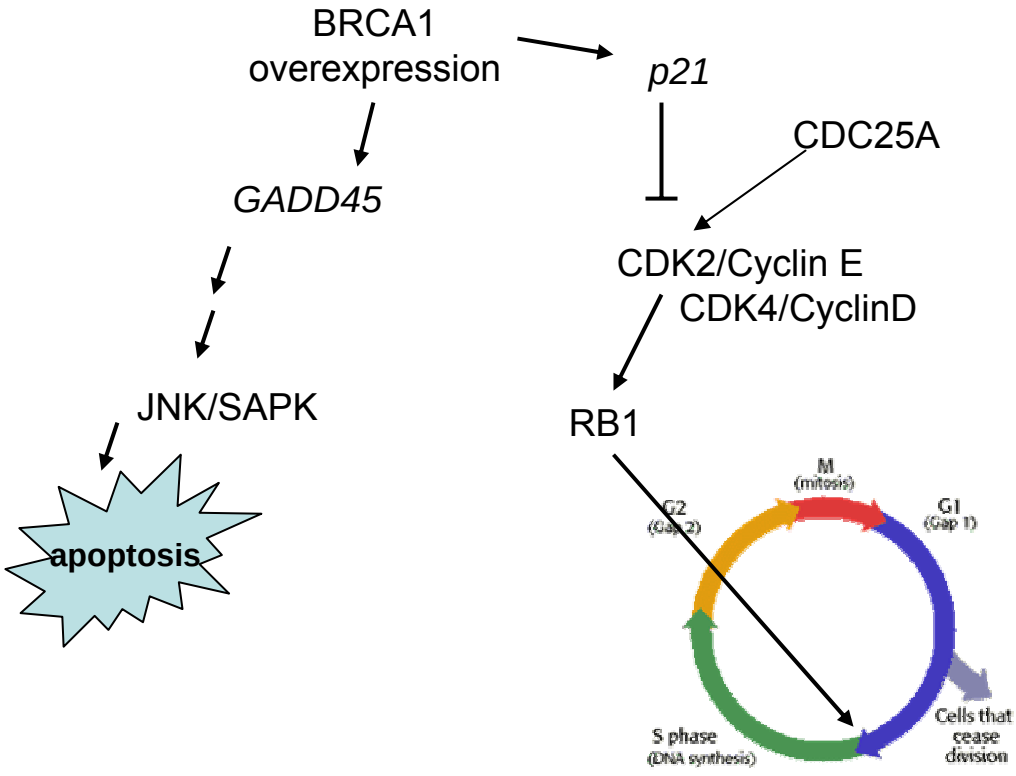


Figure 1.6: Consequences of BRCA1 overexpression at the G1/S checkpoint. BRCA1 transcriptionally upregulates *p21*. The protein product of *p21* represses the kinase activity of CDK2/CyclinE. CDK2 and CDK4 normally phosphorylate RB1 to allow progression into S phase, but repression of CDK2 activity by p21 stops progression. BRCA1 also transcriptionally upregulates *GADD45*, which is thought to lead to a JNK/SAPK-mediated apoptosis response.
Abbreviations: GADD45=Growth Arrest and DNA Damage 45, CDK=Cyclin-dependant kinase 2, RB1=Retinoblastoma protein, JNK=c-Jun N-terminal kinase, SAPK=stress-activated protein kinase.

The effect of *BRCA1* overexpression may have a second consequence, mediated through its effect on the *Growth Arrest and DNA Damage-inducible* gene *GADD45* (Harkin, 1999). *GADD45* was originally identified in a screen for genes upregulated in response to exposure to ultraviolet (UV) light, and was subsequently shown to be upregulated upon exposure to several other forms of DNA damage including hydrogen peroxide (H_2O_2), X-rays, mitomycin C (MMC), and hydroxyurea (HU) (Fornace, 1989; Papathanasiou, 1991). BRCA1 appears to interact both with the *GADD45* promoter and a region in intron 3 of *GADD45* (Harkin, 1999; Jin, 2000). However, the exact mechanism of these interactions is not fully understood, as BRCA1 might not bind directly to *GADD45* (Jin, 2000). Instead, two other proteins may serve as a link between *GADD45* and BRCA1. One is ZBRK1, a BRCA1-dependent corepressor which binds to intron 3 of *GADD45* (Zheng, 2000), another is the oncogene *C-MYC*, which has been shown to attenuate the induction of *GADD45* following UV or methyl methanesulfonate (MMS) treatments (Amundson, 1998). BRCA1, which has been shown to interact with C-MYC and represses its transactivation capabilities (likely by sequestration), is postulated to relieve this repression, and thus indirectly result in upregulation of *GADD45* following damage (Wang, 1998; Mullan, 2001). p53 is also involved in the regulation of *GADD45*, but appears to be involved primarily in the response of *GADD45* to ionizing radiation and to interact with intron 3 of the gene (Kastan, 1992).

Upregulation of *GADD45* by BRCA1 appears to be able to trigger a c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK)–mediated apoptotic response (Figure 1.6). Apoptosis is delayed from the onset of *BRCA1* overexpression, which suggests that cells arrest and then undergo apoptosis (Harkin, 1999; MacLachlan, 2000b). Other groups argue that overexpression of *BRCA1* does not result in apoptosis, although cell-cycle arrest still occurs (Randrianarison, 2001). This discrepancy may be due to the times at which the amount of apoptosis was measured, or to different levels of *BRCA1* overexpression in the various experiments (MacLachlan, 2000b). Although the relationship of BRCA1 overexpression to the induction

of apoptosis requires further work, the involvement of BRCA1 in the G1-S checkpoint is well-supported by various experiments.

1.5.3.2 BRCA1 and the G2-M checkpoint

The G2-M checkpoint delays entry into mitosis if the genome is damaged. CDC2/Cyclin B is the key kinase/cyclin complex involved in the transition into M phase. CDC2 is inhibited by phosphorylation added by the kinases WEEI and MYTI earlier in the cell cycle. Dephosphorylation of CDC2 (likely by the dephosphorylase CDC25C, although other proteins may also be involved) is both a key step in progression into mitosis and a target for inhibition during checkpoint control. The kinases ATM and ATR are again important in triggering the checkpoint; ATM activates CHK2 and ATR activates CHK1, both of which inhibit CDC25C activity to block progression into mitosis (CHK1 also activates WEEI, a direct inhibitor of CDC2) (See Figure 1.7 for a simplified diagram).

Both human (HCC1937) and murine cell lines which express only mutated BRCA1 protein fail to arrest at the G2-M boundary following γ-irradiation (Foray, 1999; Xu, 1999c; Xu, 2001a; Yarden, 2002), suggesting that BRCA1 normally plays a role in regulation of this checkpoint. Recent experiments suggest that the effect of BRCA1 is linked to the kinases CHK1 and ATM: In the absence of BRCA1, CHK1 is not activated, resulting in the deregulation of CDC2/Cyclin B and loss of control over the progression into M phase (Yarden, 2002; Yamane, 2003). Additionally, while transient expression of *BRCA1* in HCC1937 cells restores the G2-M checkpoint, transient expression of a *BRCA1* gene with a mutation in a site generally phosphorylated by the ATM kinase following DNA damage, the checkpoint is not restored, suggesting that phosphorylation of BRCA1 by ATM may be necessary for checkpoint control (Xu, 2001a).

A more recent study has also provided evidence that BRCA1 is involved in the G2-M transition and checkpoint, via an interaction with the Aurora-A kinase, which directly phosphorylates BRCA1. In a mutant mouse cell line expressing only the Δ*X.11* form of *Brca1* (this cell line lacks the G2-M checkpoint

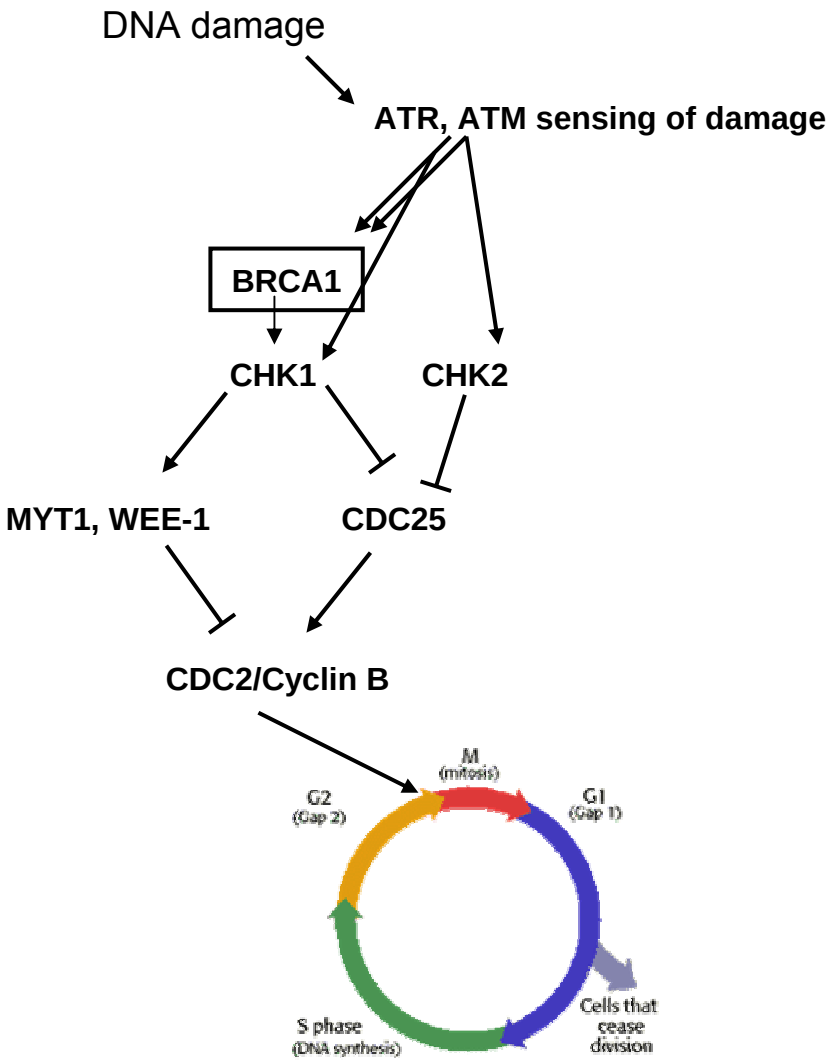


Figure 1.7: BRCA1 at the G2/M checkpoint.
Simplified schematic showing normal arrest at the G2-M boundary following DNA damage when BRCA1 is present. Loss of BRCA1 prevents activation of the CHK1 kinase, leading to deregulation of CDC2/Cyclin B and loss of G2-M checkpoint control. Following DNA damage, BRCA1-deficient cells fail to arrest at this boundary. Abbreviations: CHK1, MYT1, and WEE-1=cell-cycle dependent kinases, CDC25= cyclin-dependent kinase 25, CDC2=cell division cycle 2. Figure adapted from (Yarden, 2002) and (Iliakis, 2003).

following treatment with γ-irradiation (Xu, 1999c)), transient expression of wildtype *Brca1* restores the γ-irradiation-induced G2-M checkpoint. However, expression of a *Brca1* transgene carrying a mutation in the residue phosphorylated by Aurora-A abrogated the irradiation-induced checkpoint (Ouchi, 2004). Loss of Aurora-A itself also abrogates the G2-M checkpoint (Hirota, 2003). In summary, in both mouse and human cell lines, several experiments have provided evidence for the importance of BRCA1 in the G2-M checkpoint, although further experiments are needed to define the exact mechanism of its action.

1.5.3.3 Microarray experiments and expression of cell-cycle genes

Microarray analyses provide evidence that overexpressing *BRCA1* influences the expression profile of a number of genes, including ones involved in cell-cycle checkpoint control (Atalay, 2002; Welcsh, 2002). This type of experiment is likely to be relevant to the DNA repair phenotypes of BRCA1, as overexpression of *BRCA1* appears to occur following DNA damage (Clarkin, 2000; MacLachlan, 2000a). Microarray analyses of *BRCA1*-related tumours indicate that profiles of tumour RNA can be used to categorize breast tumours as sporadic or *BRCA1*- or *BRCA2*-related, a potentially useful diagnostic tool (Berns, 2001; Hedenfalk, 2001; Hedenfalk, 2003). However, in both types of study, the biological relevance of the genes identified is not completely clear. A number of genes which have not been previously studied in relation to BRCA1 have been identified, and additional experiments must be performed in order to clarify their involvement with BRCA1. Additionally, the small sample size in most studies makes meaningful statistical comparisons between tumour types difficult at present. Microarray technology has a very promising future, and it is expected that further studies will prove very useful in furthering the understanding of BRCA1-related biology and tumourigenesis, including its effects on cell cycle–related genes.

1.5.4 Transcriptional regulation of BRCA1

BRCA1 expression, transcription, and mRNA and protein levels appear to be influenced by a number of factors (as discussed in the previous section). At

the expression level, both positive and negative factors have been proposed to alter *BRCA1* expression levels. These include the non-histone chromatin protein HMGA1, which has been shown to bind to the promoter region of *BRCA1* and downregulate its expression, resulting in a lower amount of both mRNA and protein expression. This effect of the HMGA1 protein is confirmed by murine *Hmga1*$^{-/-}$ knockout ES cells, which have higher levels of *Brca1* mRNA expression than do wildtype cells (Baldassarre, 2003).

BRCA1 expression may be upregulated through the action of E2F1 (E2F-transcription factor-1) and RB1. The RB1 protein binds to and sequesters E2F transcription factors; normally, phosphorylation of RB1 at the G1-S boundary inactivates RB1 to allow E2F-mediated upregulation of genes needed to pass through the cycle boundary (Stevaux and Dyson, 2002). The promoters of both human and mouse *BRCA1* contain E2F1 binding sites (to which recombinant E2F1 will bind *in vitro*). Overexpression of *E2F1*, either in transgenic mice or *in vitro* in a human cell line, results in upregulation of *Brca1* mRNA expression. *Brca1* expression is also upregulated in *Rb1*$^{-/-}$ MEFs, perhaps because E2F1 is expected to be active because of loss of sequestration. Ectopic expression of the *Rb1* gene in *Rb1*$^{-/-}$ MEFs restores the expression level of *Brca1* (Wang, 2000a).

p53 also appears to participate in the regulation of BRCA1 (MacLachlan, 2000a). Following DNA damage, BRCA1 mRNA and protein levels are rapidly upregulated (by 15 minutes post-damage), but fall again by 4-12 hours post-damage (Andres, 1998; Clarkin, 2000). This reactive downregulation appears to be caused by p53 binding to the promoter of *BRCA1*. In cancer cell lines which lack p53, damage-induced upregulation of *BRCA1* occurs, but suppression after the initial burst of expression does not occur (MacLachlan, 2000a). In addition, overexpression of *BRCA1* may stabilize the p53 protein; two different regions of BRCA1 appear to interact with p53 (see Figure 1.5 and section 1.5.3.1). Taken together, these results suggest that BRCA1 and p53 may be involved in a type of feedback loop (Somasundaram, 1999). These data provide evidence that BRCA1 and p53 participate in at least one common pathway. However, the fact that *BRCA1*-related tumours frequently

carry *p53* mutations (Table 1.2) suggests that these two proteins have additional roles in separate pathways.

1.6 INTERACTIONS WITH OTHER PROTEINS

Numerous experiments have provided evidence that BRCA1 interacts with many proteins involved in gene regulation, the cell cycle, and/or DNA repair (Figure 1.5). In fact, one group has asserted that BRCA1 associates with a large number of DNA-repair proteins in a BASC (BRCA1-associated genome surveillance complex). Characterization of BASC components has identified such proteins as the RAD50-MRE11-NBS1 complex thought to play a role in double-strand break repair (DSBR), meiotic recombination and maintenance of telomeres, as well as components of the mismatch-repair system and the Bloom's Syndrome protein (BLM), which confers cancer predisposition through a high propensity for sister-chromatid exchanges and mitotic recombination with resultant LOH (Ellis, 1995; Wang, 2000b; Thompson and Schild, 2002).

One caveat to the large number of published interactions is that a diverse array of methods have been used to demonstrate these interactions, and not all partnerships may prove to be biologically relevant when more stringently investigated. Some well-characterized interactions are given as examples here; further cases are discussed throughout this introduction. BRCA1 appears to interact with BRCA2 (Chen, 1998). Both BRCA1 and BRCA2 interact with RAD51 (although this may be an indirect interaction in the case of BRCA1), the mammalian homologue of the bacterial RecA protein which mediates strand-exchange during recombination (Shinohara, 1993; Scully, 1997c). All three co-localize to S phase and DNA damage-induced nuclear foci, and may act in a common pathway during crossing over in meiosis (Chen, 1998; Chen, 1999). BRCA1 also interacts with RAD50 and the Nijmegen breakage syndrome protein NBS1, both part of the RAD50-MRE11-NBS1 complex (Varon, 1998; Wang, 2000b). RAD50 and BRCA1 also co-localize in some damage-induced nuclear foci (Haber, 1998; Petrini, 1999; Zhong, 1999; Wang, 2000b), as does RAD51. However, while RAD50 or

RAD51 alone may co-localize with BRCA1 in foci, the former two proteins are rarely found in the same focus (Maser, 1997; Zhong, 1999). Interestingly, homozygous mouse knockouts of *Brca1*, *Brca2*, *Rad50*, or *Rad51* all exhibit early embryonic lethality with similar phenotypic profiles (Lim and Hasty, 1996; Ludwig, 1997; Sharan, 1997; Luo, 1999).

1.6.1 The RING domain

The N-terminus of BRCA1 contains a cysteine-rich, Zn-finger motif with a C3-H-C4 configuration (C=cysteine and H=histidine). This motif is known as a RING finger, after RING1 (Really Interesting New Gene 1), the first novel protein identified which carried the motif (Freemont, 1991). Originally, this Zn-finger motif was thought to be involved in binding DNA (Lovering, 1993), but proteins containing the RING-finger are now known to be one of two major types of E3 ubiquitin ligases, involved in ubiquitination of proteins targeted for destruction through the proteasome-mediated degradation pathway (Pickart, 2001; Ravasi, 2003; Semple, 2003). This pathway consists of three steps (Figure 1.8). An E1 enzyme activates the small protein ubiquitin and interacts with one of several E2s, which carry the activated ubiquitin and interface in turn with an E3 protein. E3s catalyze the transfer of ubiquitin from an E2 to a protein substrate. E3s recognize only one substrate, or several closely-related substrates. Generally, a chain of several ubiquitin molecules (polyubiquitination) is assembled on proteins prior to their degradation by the 26S proteasome (Weissman, 2001).

1.6.2 BARD1 is an important RING-binding partner of BRCA1

The RING domain of BRCA1 is the interaction site for BARD1 (BRCA1-Associated RING Domain 1), an important protein partner of BRCA1. BARD1 resembles BRCA1, in that it has an N-terminal RING domain and two C-terminal BRCT repeats, but is smaller (777 aa), and also contains ankyrin repeats (Wu, 1996). The mouse homologue of BARD1, Bard1, shares a 77% overall identity with its human counterpart, with high conservation at the RING domain, BRCT repeats, and ankyrin repeats. Though the higher overall

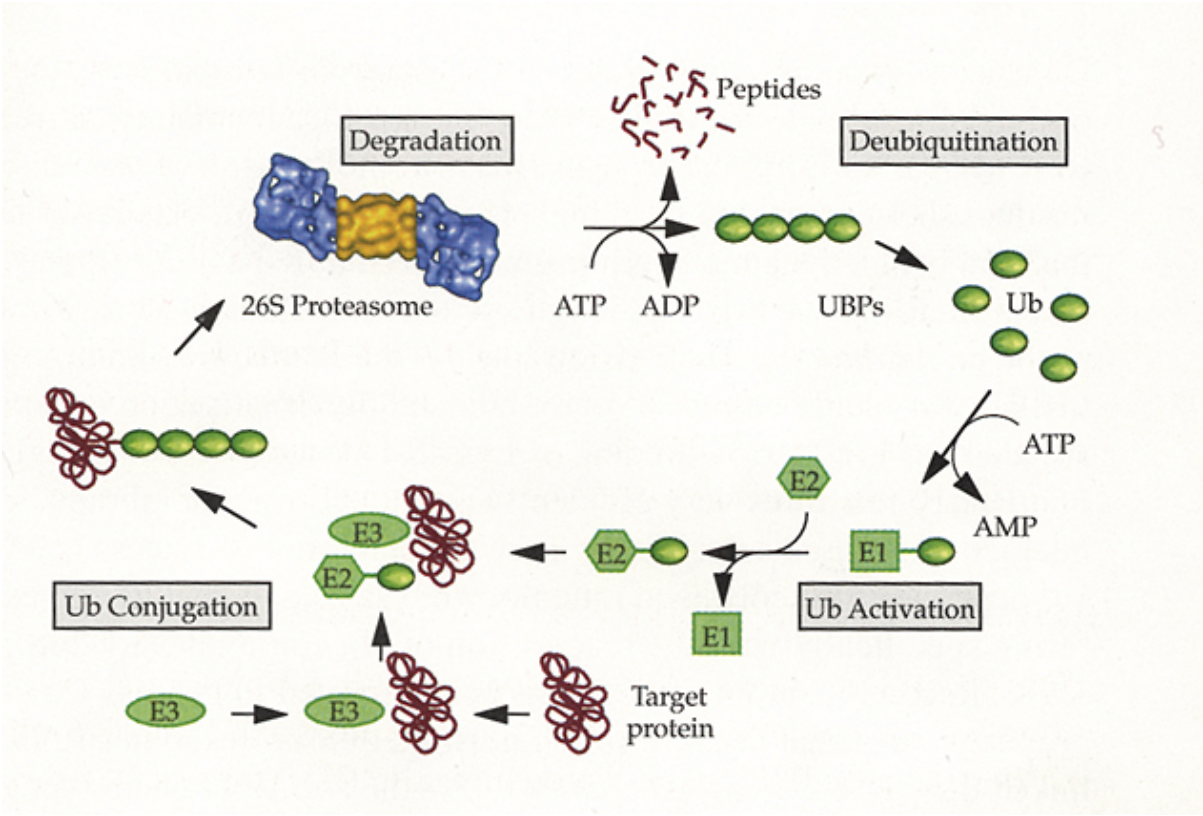


Figure 1.8: The proteasome-mediated ubiquitination pathway of protein degradation. Ubiquitin molecules (small green ovals) are activated by the E1 enzyme, then activated ubiquitin is passed to an E2 enzyme. E3 ubiquitin ligases interface with a single protein target (or closely-related group of targets), and catalyze transfer of the activated ubiquitin molecule from the E2 enzyme to the target protein. A polyubiquitin chain marks the target protein for proteasome-mediated degradation. Recycling of ubiquitin molecules follows protein degradation. Ub=ubiquitin.
Figure drawn by Colin Gordon (see Gordon in references).

homology seems to indicate that BARD1 is more highly conserved than BRCA1, BARD1 is much smaller than BRCA1. If the highly homologous BARD1 regions are not counted, the overall BARD1-Bard1 identity is only 61%, much closer to the 57% overall identity of BRCA1 and Brca1 (Ayi, 1998). Mouse Bard1 and Brca1 also interact via their RING domains (Ayi, 1998).

Polypeptides containing the BRCA1 or BARD1 RING domains have been used to demonstrate that both proteins will form homodimers *in vitro*, although heterodimerization appears to be preferred (Brzovic, 1998; Meza, 1999). Recently, it was shown that BARD1 and BRCA1 RING domains will assemble into dimers and tetramers, but are more commonly observed in supramolecular structures containing 12 RING domains. Whether these larger assemblies of BRCA1 and BARD1 are normally present in cells is unclear, as full-length BRCA1 was not used in these experiments, and the reactions were performed *in vitro* using purified proteins. These studies also indicated that BRCA1 or BARD1 alone will form 12-RING bodies, but the cancer-related C64G mutation in the BRCA1 RING domain abolishes the ability to form 12-RING bodies (Kentsis, 2002).

1.6.3 E3 ubiquitin ligase activity of BRCA1/BARD1

Alone, both BRCA1 and BARD1 do have some E3 ubiquitin ligase activity, but it is low compared to the activity of BRCA1 and BARD1 together. Additionally, the BRCA1/BARD1 12-RING bodies described above are a more effective E3 ligase than BRCA1/BARD1 dimers or tetramers (as measured by an *in vitro* ubiquitination assay using polyubiquitin-specific antibodies), possibly because additional E2s can be conjugated to the 12-RING bodies (Hashizume, 2001; Kentsis, 2002). The BRCA1/BARD1 E3 does have characteristics of normal E3 ligases: its activity is dependent on the presence of an E2 enzyme (in their case, Ubc5c (Hashizume, 2001; Mallery, 2002)), as well as ATP, ubiquitin, and a functional E1 enzyme (Kentsis, 2002). The RING domain of BRCA1 is also the site for interaction with a ubiquitin hydrolase, or deligase, called

BAP1 (BRCA1 Associated Protein 1), but very little is known about the biological consequences of this interaction (Jensen and Rauscher, 1999).

1.6.4 Targets of the BRCA1/BARD1 E3 ubiquitin ligase

The biological targets of the E3 activity of BRCA1/BARD1 are not well-characterized as yet, though *in vitro* studies have demonstrated that the two can monoubiquitinate histone proteins such as H2AX, H2A, H2B, H3, and H4 (Chen, 2002; Mallery, 2002). The biological consequences of this monoubiquitination are not fully understood, but as monoubiquitinated proteins do not seem to be targeted to the 26S proteasome for degradation, this modification may be a way of promoting protein-protein interactions between the modified histones and chromatin-remodeling complexes (Jason, 2002).

Until recently, the Fanconi anaemia protein FANCD2 was considered a likely target for the BRCA1/BARD1 E3 ligase. Fanconi anaemia, a human syndrome characterized by cancer predisposition, is caused by defects in one of a number of *FANC* genes (D'Andrea and Grompe, 2003). BRCA1 has been shown to interact with at least two of the FANC proteins, FANCA and FANCD1 (also known as BRCA2) (Folias, 2002; Howlett, 2002). Mutation of a *FANC* gene confers susceptibility to DNA damage. Following DNA damage, FANCD2 is activated by monoubiquitination and co-localizes with BRCA1 in foci at sites of DNA damage (Garcia-Higuera, 2001; D'Andrea and Grompe, 2003). In HCC1937 cells (a human breast cancer cell line which carries only a mutated version of BRCA1 (Tomlinson, 1998)), there is a decrease in monoubiquitinated FANCD2 following γ-irradiation, which suggests that BRCA1 may play some role in this modification (Garcia-Higuera, 2001). However, it has been shown that, at least *in vitro*, BRCA1/BARD1 is unable to ubiquitinate FANCD2 (Vandenberg, 2003). More recently, a new candidate E3 has been identified, the product of the gene *PHF9*, which is mutated in some Fanconi anaemia patients and may represent a new *FANC* gene (Meetei, 2003). BRCA1 may still play some role in the ubiquitination of FANCD2 following DNA damage, but more work is needed to define this role.

Recent studies of the effects of DNA damage on RNA Polymerase II (RNA Pol II), the polymerase responsible for transcription of coding genes to mRNA, demonstrate that following UV exposure or α-amanitin treatment (to inhibit RNA Pol II activity), a percentage of the large subunit of RNA Pol II is ubiquitinated and degraded (Bregman, 1996; Nguyen, 1996; Ratner, 1998). There has been much speculation that BRCA1/BARD1 is the E3 ligase which ubiquitinates RNA Pol II, though no data have yet been published to support this claim. Recent papers have introduced other candidates: the von Hippel-Lindau protein (pVHL) ubiquitinates the phosphorylated fraction of RNA Pol II large subunit in a UV-dependent manner (Kuznetsova, 2003), as does the human protein NEDD4L, both *in vitro* and *in vivo*. Additionally, the yeast homologue of NEDD4L, Rsp5, ubiquitinates yeast RNA Pol II (Beaudenon, 1999). Although the existence of these alternative candidates does not exclude the possibility that BRCA1/BARD1 can ubiquitinate RNA Pol II, it does suggest that other E3 ligases may have the same ability.

In fact, the only *in vivo* activity of the BRCA1/BARD1 E3 identified to date appears to be auto-polyubiquitination; that is, heterodimerization appears to catalyze the assembly of polyubiquitin chains on BRCA1 and BARD1 themselves (Chen, 2002). Polyubiquitination of BRCA1/BARD1 appears to increase the E3 ligase activity of the heterodimer (Mallery, 2002). This auto-polyubiquitination seems to contradict two reports that BRCA1 and BARD1 stabilize one another (Joukov, 2001b; McCarthy, 2003), since polyubiquitinated proteins are normally targeted for destruction, but a recent paper shows that the polyubiquitin chains assembled on BRCA1/BARD1 have an unconventional Lys-6 linkage configuration, different from the commonly-observed Lys-48 linkage of polyubiquitin chains on proteasome-targeted proteins (Wu-Baer, 2003). Furthermore, a recent *in vitro* study demonstrated that polyubiquitinated BRCA1/BARD1 is de-ubiquitinated – but not degraded – by the 26S proteasome (Nishikawa, 2004). While little is known about alternative consequences of protein ubiquitination, proteasome targeting is not the sole reason for ubiquitin modification, and the ubiquitin chains on BRCA1/BARD1 may serve to stabilize the proteins, direct them to other

proteases, or alter their conformation to facilitate interactions with other proteins (Chen, 2002; Aguilar and Wendland, 2003; Schnell and Hicke, 2003).

1.6.5 BARD1 alone

Several studies indicate that the majority of cellular BARD1 is associated with BRCA1 (Yu and Baer, 2000; Joukov, 2001b). Biochemical fractionation experiments show that a percentage of BARD1 does not co-purify with BRCA1, which suggests that BARD1 may have BRCA1-independent cellular functions (Chiba and Parvin, 2002), although few studies of these putative independent functions have been published. Based on its interaction with BRCA1, it is hypothesized that BARD1 might be a tumour suppressor itself. However, screening panels of tumours (mainly breast tumours) for BARD1 mutations has indicated that if BARD1 is involved in tumourigenesis, its involvement is either rare or confined to cancers which have not been investigated (Thai, 1998; Ghimenti, 2002; Ishitobi, 2003). A mouse knockout of *Bard1* has been generated; it is phenotypically identical to both *Brca1* knockout and *Brca1/Bard1* double knockout mice, although the embryonic lethality of both models precludes extensive investigation (McCarthy, 2003). Mouse models will be discussed further in section 1.9.

1.7 NUCLEAR LOCALIZATION: NLS, NES, AND BARD1

As described in section 1.5.2, both mouse and human *BRCA1* express natural splice isoforms which lack all or most of exon 11, the largest exon. Transiently overexpressed human BRCA1 ΔX.11 or ΔX.11b proteins are localized in the cytoplasm, unlike the full-length form which is found in the nucleus and cytoplasm (Thakur, 1997; Wilson, 1997). Examination of exon 11 following these overexpression experiments revealed the presence of two nuclear localization sequences (NLSs) at the 5' end (Thakur, 1997; Wilson, 1997) (Figure 1.9).

Murine *Brca1* also has NLSs in exon 11, identical in sequence to those of human *BRCA1* (Figure 1.9) (Xu, 1999c; Bachelier, 2000). Transient

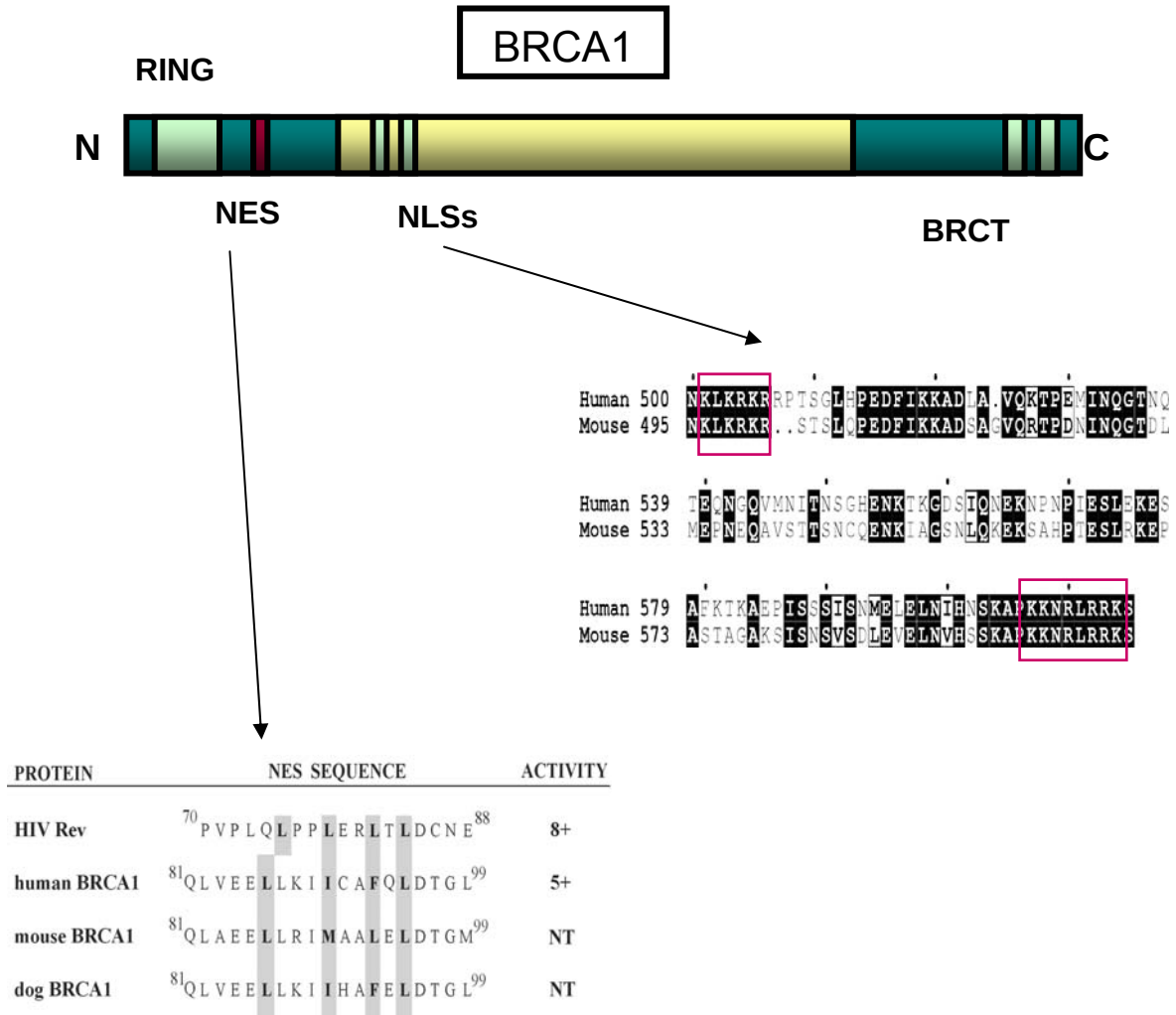


Figure 1.9: Conservation of the NLS and NES signals. BRCA1 protein schematic showing the nuclear localization sequences (NLSs) and the nuclear export sequence (NES). The alignment of the mouse and human NESs with the HIV Rev protein is taken from (Rodriguez, 2000). Mouse and human NLS alignments (red box) demonstrate that these domains are absolutely conserved. The yellow box indicates the part of the protein coded by exon 11. Both the RING domain and the BRCT repeats are indicated in green.
Abbreviations: HIV=human immunodeficiency virus, NES=nuclear export signal, NLSs=nuclear localization signals.

overexpression of *Brca1 ΔX.11 in vitro* revealed that the resulting Brca1 ΔX.11 protein was found in the cytoplasm, similar to what is observed in human cells overexpressing *BRCA1 ΔX.11*. In contrast, in a MEF cell line homozygous for the *Brca1 ΔX.11* isoform, in which the Brca1 ΔX.11 protein is expressed at physiological levels, Brca1 ΔX.11 protein is found in the nucleus, and even forms S phase nuclear foci (Xu, 1999c; Bachelier, 2000).

A nuclear export signal (NES) in exon 6 of *BRCA1* has also been described. This signal was identified based on its similarity to the export signal of the human immunodeficiency virus (HIV) Rev protein, and is well-conserved between species (Figure 1.9). When joined to a reporter gene, the *BRCA1* NES will stimulate nuclear export of the reporter protein (Rodriguez and Henderson, 2000). Overexpression of a mutant *BRCA1* transgene either lacking the NLS or carrying a mutated NES (with two key residues mutated) results in almost exclusively nuclear localization of BRCA1 (Rodriguez and Henderson, 2000). However, if the NES and NLSs are the only factors involved in localization, then a mutant transgene which retains both NES and NLSs should have the same localization pattern as full-length BRCA1. This is not the case. Fabbro *et al.* demonstrated that the majority of cells overexpressing a *BRCA1* transgene which lacks the 5' RING domain (but includes both NLSs and the NES) only showed cytoplasmic localization of BRCA1 (BRCA1 was seen in both the cytoplasm and nucleus in some cells), in contrast to cells expressing full-length *BRCA1*, in which the protein was detected in the nucleus and cytoplasm (Figure 1.10).

Based on these data, Fabbro *et al.* postulated that the binding of another protein to the BRCA1 RING domain might mask the NES (Fabbro, 2002). While testing the effect of RING-binding proteins on the localization of BRCA1, they found that in cells co-transfected with a full-length *BARD1* transgene and a *BRCA1* transgene carrying a mutated NLSs, BRCA1 was located predominantly in the nucleus. When a *BARD1* transgene was co-transfected with a wildtype *BRCA1* transgene, more BRCA1 was localized in the nucleus than when *BARD1* was not overexpressed (Figure 1.10). This increase in nuclear localization was abrogated on deletion of the RING

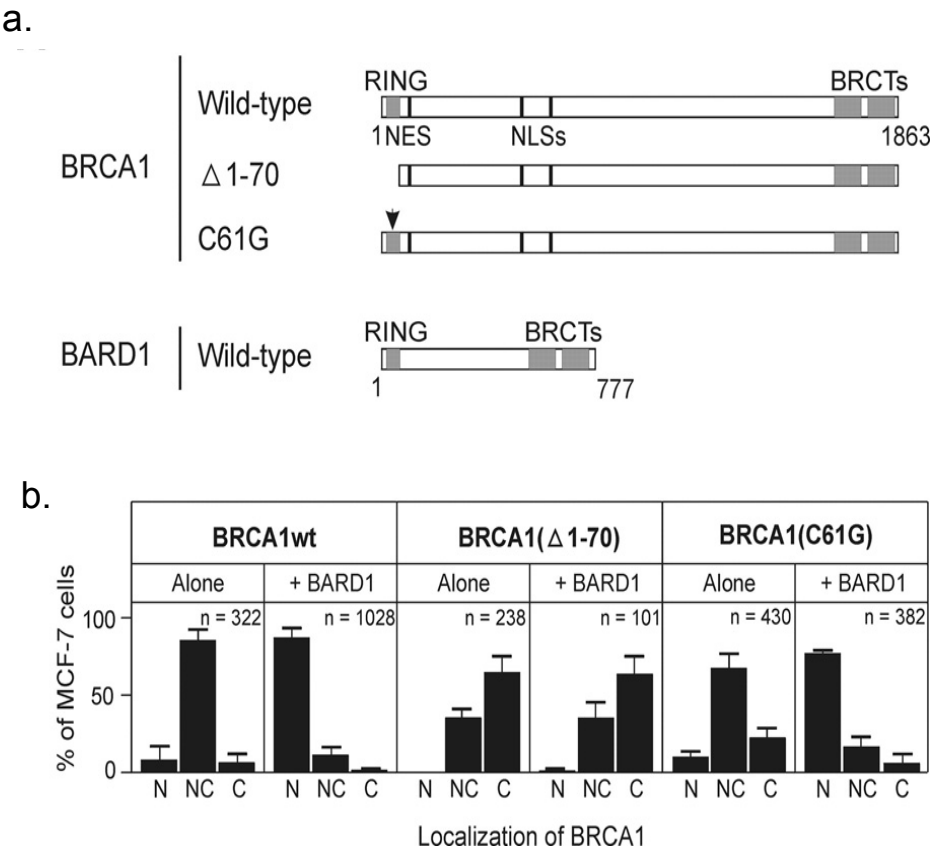


Figure 1.10: Nuclear localization of BRCA1 in the presence or absence of localization signals and BARD1. a. BRCA1 and BARD1 transgenes used in this study. **b.** Percentage of transfected cells showing nuclear (N), nuclear and cytoplasmic (NC), or cytoplasmic (C) localization of BRCA1 protein, as detected by immunofluorescence. Transgenes were transfected with or without a BARD1 transgene ("+BARD1" or "Alone"). The first panel shows the localization of wildtype BRCA1, the second of a transgene lacking the RING domain at the N-terminus (Δ1-70), and the third of a transgene with a cancer-related mutation in the BRCA1 RING domain which still allows binding to BARD1 (C61G).
Figures taken from (Fabbro, 2002).

domain of either protein (Fabbro, 2002). The published BRCA1-BARD1 RING-interaction structure confirms that the NES is buried when BARD1 binds to BRCA1 (Brzovic, 2001b). The hypothesis that BARD1 is a nuclear chaperone and/or nuclear retention partner of BRCA1 may help to explain why overexpressed BRCA1 ΔX.11 protein is located in the cytoplasm, while Brca1 ΔX.11 protein expressed at endogenous levels has the expected nuclear-and-cytoplasmic localization pattern: endogenous levels of BARD1 may be insufficient to cope with the overexpressed transgene (Thakur, 1997; Wilson, 1997; Xu, 1999c; Bachelier, 2000). It cannot be ruled out that the mutated proteins arising from the transgenes used by Fabbro *et al.* may not have folded properly, but the proteins were detected at the expected levels, and N-terminal truncations of BRCA1 have been used in *in vitro* studies in the past, most notably during confirmation of the BRCA1-BARD1 interaction (Wu, 1996). Additionally, it is possible that other proteins which interact with BRCA1 at other domains may be able to serve as chaperones. A very recent paper has also provided evidence that intact BRCA1 is necessary for entry of BARD1 into the nucleus. As is the case for BRCA1, binding of BARD1 to BRCA1 at the RING domain buries the BARD1 NES, resulting in retention of the BARD1 protein in the nucleus. These authors have also provided evidence that increasing the cytoplasmic BARD1 fraction may lead to increased levels of apoptosis (Rodriguez, 2004).

The role of BARD1 in the nuclear localization of BRCA1 does not mean that the NLSs are unimportant or non-functional; in fact, they appear to augment BARD1-mediated import of BRCA1. In the absence of the RING domain, full-length BRCA1 is localized in both the nucleus and the cytoplasm, which indicates that the NLSs are able to influence nuclear import of BRCA1 (Fabbro, 2002).

1.8 BRCA1 IS A TRANSCRIPTION FACTOR

1.8.1 Transactivation *in vitro*

BRCA1 was originally thought to be a potential transcription factor, based on its N-terminal Zn-finger domain and acidic C-terminus (Miki, 1994). The C-terminus of BRCA1 (including the BRCT repeats) is able to function as a transactivation domain. A fusion of this region with the GAL4 DNA-binding domain will activate a reporter gene fused to the GAL4 activation domain (Chapman and Verma, 1996; Monteiro, 1996). However, *in vitro* studies show that a BRCA1 polypeptide containing the RING domain does not bind to double- or single-stranded DNA-cellulose columns. This BRCA1 polypeptide is likely to be folded correctly, as it will heterodimerize with a BARD1 RING domain polypeptide (Meza, 1999). However, residues 452-1079 in the middle of BRCA1 will bind DNA. This reaction does not appear to depend on sequence specificity, but branched substrates are preferred over linear ones, and longer sequences (over 500 bp) are preferred over shorter ones (Paull, 2001). The BRCT repeats at the C-terminus also appear to be able to bind to linear DNA in a sequence-independent manner (Yamane and Tsuruo, 1999). BRCA1 may protect bound DNA from the exonuclease activity of recombinant MRE1, whether MRE1 is part of a recombinant RAD50-MRE1-NBS1 complex or alone. This apparent protection suggests that BRCA1 binds to DNA as part of a repair process, not a transactivation process (Paull, 2001). In contrast to data which suggest that BRCA1 binds to DNA in a sequence-independent manner, a recent study demonstrates that BRCA1 appears to exist in a transcriptional regulatory complex in the cell which binds to a specific sequence motif found in such genes as *GADD45, STAT5A*, and the gene coding for Cyclin B1 (*CCNB1*). A reporter gene carrying this sequence motif was upregulated upon transfection of a *BRCA1* transgene, but expressed at a higher level following transfection of a *BRCA1* transgene carrying a cancer-related mutation (Cable, 2003).

1.8.2 Transcription factor activity *in vivo*

BRCA1 also appears to act as a transcription factor *in vivo*, although in many cases, its influence may depend on other proteins. For example, BRCA1 may act as a p53-dependent transcriptional activator, upregulating such p53-responsive genes as *BCL-associated X (BAX), Mouse Double-Minute 2 human homologue* (*HDM2*), and *p21* (Ouchi, 1998; Zhang, 1998).

BRCA1 also upregulates *p21* independently of p53 (Somasundaram, 1997), an influence which appears to be enhanced further upon overexpression of the androgen receptor (AR) (Park, 2000; Yeh, 2000) and repressed by overexpression of the transcriptional co-repressors CtBP/CtIP (Li, 1999). BRCA1 is thought to physically interact with both CtIP and the AR (Figure 1.5) (Wong, 1998; Li, 1999; Park, 2000; Yeh, 2000). Upregulation of *p21* expression is expected to have an effect on cell-cycle regulation, as p21 inhibits the kinase CDK2 (el-Deiry, 1993; Harper, 1993), which phosphorylates the protein RB1, a key event in the G1-S transition (see Figure 1.6). Indeed, overexpression of BRCA1 *in vitro* appears to result in dephosphorylation of RB1 (Somasundaram, 1997; MacLachlan, 2000b).

BRCA1 also may inhibit oestrogen-mediated signaling through the oestrogen receptor ER-α (Fan, 1999). Considering the high rate of loss of activity of oestrogen receptors in mouse and human *BRCA1*-related tumours (Table 1.2), this suggests that the ER has roles in other cellular pathways, otherwise loss of ER activity would not be expected to contribute to *BRCA1*-related tumourigenesis (Loman, 1998; Lakhani, 2002). The interaction of BRCA1 and the ER is likely to be complex, as it appears to be abolished by other proteins such as p300, and does not happen at all in some cell lines (Fan, 2001; Fan, 2002).

1.8.3 Other indications that BRCA1 may be linked to transcriptional control

Several publications provide evidence that BRCA1 is linked to the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway, an important set of transcriptional regulators involved in the response to cytokines or growth factors (Aaronson and Horvath, 2002). BRCA1 may directly interact with JAK1 and JAK2, and overexpression of *BRCA1* has been shown to result in constitutive activation of STAT3 in a prostate cancer cell line (Gao, 2001), as well as upregulation of *JAK1* and *STAT1* in a human embryonic kidney cell line (Welcsh, 2002).

Biochemical fractionation experiments demonstrate that BRCA1 and BARD1 co-purify with the RNA Pol II holoenzyme (Scully, 1997a; Neish, 1998; Chiba and Parvin, 2002), a polymerase complex including RNA Pol II and basal transcription factors such as TFIIH. Depending on the purification method, this complex may also include DNA repair factors such as RAD51, the Ku heterodimer, and replication protein A (Maldonado, 1996), but not sequence-specific transcription factors (Ossipow, 1995). BRCA1 and BARD1 have been shown to interface with the holoenzyme though an interaction with RNA Helicase A (Anderson, 1998). However, recent work has indicated that BRCA1 appears to associate with the hyper-phosphorylated form of RNA Pol II, the processive form of the enzyme, suggesting that BRCA1 may have a role in transcriptional elongation rather than (or in addition to) initiation (Krum, 2003). Further studies are still needed to define the consequences of the interaction of BRCA1 and RNA Pol II.

BRCA1 and BARD1 may also be involved in a DNA-damage–induced transcription block. BARD1 interacts with CstF-50, a subunit of the cleavage-stimulation-factor complex which plays a role in the 3' end cleavage of mRNA precursors prior to polyA tail synthesis (Kleiman and Manley, 1999). UV light exposure or a DNA replication block induced by HU treatment results in a temporary block in cleavage, during which the amount of cellular CstF50/BRCA1/BARD1 complex increases. BARD1 is likely to be necessary

for this interaction, as no damage-induced elongation block occurs if BARD1 is mutated (Kleiman and Manley, 2001).

A few experiments have suggested links between BRCA1 and chromatin proteins and/or the chromatin remodeling complex. BRCA1 appears to interact with a component of the SWI/SNF chromatin-remodeling complex (BRG1), suggesting a possible mechanism for transcriptional activation (Bochar, 2000), and it also interacts with a DEAH-type helicase called BACH (BRCA1-associated C-terminal helicase), which may play a role in DSBR (Cantor, 2001). The phosphorylated version of the chromatin protein H2AX co-localizes with BRCA1 after DNA damage (Chen, 2002). Yeast two-hybrid screens have provided limited evidence for an interaction between BRCA1 and the histone deacetylase proteins HDAC1 and HDAC2 (Yarden and Brody, 1999). As discussed earlier, HMGA1, a non-histone chromatin protein, may downregulate BRCA1 (Baldassarre, 2003). There is also evidence that BRCA1 may have a role in the organization of the non-coding RNA *Xist* on the inactivated X-chromosome (Ganesan, 2002). While the interaction of BRCA1 with chromatin remodeling proteins may be an indication of a physical linkage of BRCA1 with chromatin remodeling, the potential functional consequences of these interactions require further study.

1.9 BRCA1 MOUSE KNOCKOUT MODELS

1.9.1 Knockout alleles of *Brca1*

Although much has been learned about *BRCA1* through studying human cell lines, tumours, and breast cancer families, modeling *BRCA1*-related breast cancer in the mouse was expected to reveal the functions of the gene and allow tumourigenicity to be carefully studied. To date, several *Brca1* mouse knockout models have been generated (Table 1.3). For those knockouts which generate functionally null alleles, embryonic lethality occurs in homozygous mutants between embryonic day (E) 5.5- E10.5. Heterozygotes are normal and healthy with no increased predisposition to cancer, and double-targeted ES cells cannot be generated (Hakem, 1996; Liu, 1996;

Table 1.3: Mouse *Brca1* knockouts.

#	Description of allele	Phenotype of homozygous mutation	Secondary mutations or backgrounds	Other phenotypes/ Results of tumourigenesis studies	References
1	Deletion of exon 2 (part of the RING domain)	Embryonic lethality by E5.5-E9.5	*Brca2*$^{-/-}$ or *Bard1*$^{-/-}$ background: no change in *Brca1*$^{-/-}$ phenotype. *p53*$^{-/-}$background: *Brca1*$^{-/-}$ embryonic development extended by one day.	*Brca1*$^{+/-}$: no increased predisposition to tumourigenesis compared to wildtype mice.	(Ludwig, 1997; McCarthy, 2003)
2	Deletion of exons 5 and 6	Embryonic lethality by E7.5	*p21*$^{-/-}$ or *p53*$^{-/-}$backgrounds: either extended *Brca1*$^{-/-}$ embryonic development by one day.	*Brca1*$^{+/-}$: no increased predisposition to tumourigenesis compared to wildtype mice.	(Hakem, 1996; Hakem, 1997)
3	Conditional, *loxP*-flanked deletion of exons 5 and 6	Viable mice	T-cell specific Cre.	Decreased proliferation of T-cells in *Brca1*$^{c/c}$; T-cell Cre mice.	(Mak, 2000)
4	Deletion of part of 5' end of exon 11 and part of intron 10 (ΔX.11)	Embryonic lethality by E8.5		*Brca1*$^{+/-}$: no increased predisposition to tumourigenesis compared to wildtype mice.	(Liu, 1996)
5	Deletion of part of 5' end of exon 11 and part of intron 10 (ΔX.11)	Embryonic lethality between E10.5- E 13.5	*p53*$^{-/-}$ background (for tumourigenesis studies).	*Brca1*$^{+/-}$: no increased predisposition to tumourigenesis compared to wildtype mice. *Brca1*$^{+/-}$; *p53*$^{-/-}$: tumour latency and spectrum not different from *p53*$^{-/-}$ mice; 20 of 22 tumours screened retained a wildtype allele of *Brca1*.	(Gowen, 1996; Cressman, 1999)
6	Deletion of part of 5' end of exon 11 and part of intron 10 (ΔX.11)	Embryonic lethality between E7.5- E9.5		*Brca1*$^{+/-}$: no increased predisposition to tumourigenesis compared to wildtype mice.	(Shen, 1998)
7	Deletion of exon 11 (Cre-excision of *loxP*-flanked exon in ES cells; ΔX.11)	Embryonic lethality between E12.5-E18.5, although ~2% of homozygotes survive to adulthood.	*p53*$^{+/-}$ or *p53*$^{-/-}$ background (for tumourigenesis studies).	18/66 (27%) *Brca1*$^{-/-}$; *p53*$^{+/-}$ mice developed thymic lymphomas by 28 weeks; all had lost wildtype allele of *p53*. 14/14 (100%) *Brca1*$^{-/-}$; *p53*$^{-/-}$ developed thymic lymphomas by 15 weeks.	(Xu, 1999b; Xu, 2001; Bachelier, 2003)

Table 1.3: Mouse *Brca1* knockouts, continued.

Abbreviations: Apc^{min}: adenomatous polyposis coli (multiple intestinal neoplasia); *K-14: Keratin-14*; *K-5: Keratin-5*; MMTV: mouse mammary tumour virus-long terminal repeat promoter; *WAP*: whey acidic protein, gene promoter; c: conditional allele; E: embryonic day.

#	Description of allele	Phenotype of homozygous mutation	Secondary mutations or backgrounds	Other phenotypes/ Results of tumourigenesis studies	References
8	Conditional, *loxP*-flanked deletion of exon 11	Viable mice	MMTV-Cre, *WAP*-Cre, or *K5*-Cre transgenes. $p53^{+/-}$ or *K5-E2F1* backgrounds (for tumourigenesis studies).	$Brca1^{c/c}$; Cre or $Brca1^{-/c}$; Cre: 35 of 150 mice developed tumours by 2 years. $Brca1^{c/c}$; $p53^{+/-}$; Cre or $Brca1^{-/c}$; $p53^{+/-}$; Cre: almost all of 56 animals developed tumours by 15 months. Most had lost the second *p53* allele. $Brca1^{-/c}$; *K5*-Cre: 13 of 18 mice developed tumours, mostly oral epithelial or inner ear canal by 20 months. $Brca1^{-/c}$; *K5*-Cre; *K5-E2F1*: tumourigenesis accelerated over latter class. Skin or reproductive tract tumours (only 5 mice).	(Xu, 1999a; Brodie, 2001; Berton, 2003)]
9	Exon 11 truncation mutation (*tr*)	Viable mice, males infertile.	$p53^{+/-}$ or $p53^{-/-}$ backgrounds (for tumourigenesis studies). Background strain determined percentage of viability.	$Brca1^{tr/tr}$: wide range of tumours developed in 76 of 86 mice, including 12 mammary tumours. Mean latency ~17 months. $Brca1^{tr/tr}$; $p53^{+/-}$ or $Brca1^{tr/tr}$; $p53^{-/-}$: possible acceleration of *p53*-related tumourigenesis, very small cohort.	(Ludwig, 2001)
10	Conditional, *loxP*-flanked deletion of exons 9-13	Viable	*p53* conditional background (*loxP*-flanked deletion of exons 2-10) and *K14*-Cre transgene.	$Brca1^{c/c}$; *K-14*-Cre; $p53^{c/c}$: 11 of 11 mice developed tumours, mean latency 6 months, no wildtype *p53* or *Brca1* detected. $Brca1^{+/c}$; *K-14*-Cre; $p53^{c/c}$: 8 of 8 mice developed tumours, mean latency 11 months. No wildtype *p53* detected, but all tumours had at least one wildtype *Brca1* allele.	(Jonkers, 2003)
11	Deletion of exons 20-24 (last BRCT repeat)	Embryonic lethality by E10.5	$p53^{+/-}$, $p53^{-/-}$, or $Apc^{min/+}$ backgrounds (for tumourigenesis studies).	$Brca1^{+/-}$: no increased predisposition to tumourigenesis compared to wildtype mice. $Brca1^{+/-}$; $p53^{+/-}$ or $Brca1^{+/-}$; $p53^{-/-}$: tumorigenesis not accelerated compared to $p53^{+/-}$ or $p53^{-/-}$ alone. $Brca1^{+/-}$; $Apc^{+/min}$: tumorigenesis not accelerated compared to $Apc^{+/min}$ alone.	(Hohenstein, 2001)

Ludwig, 1997; Shen, 1998; Hohenstein, 2001). Embryonic lethality appears to result from growth suppression and not from apoptosis (Hakem, 1996; Liu, 1996; Xu, 1999c), although embryos homozygous for one mutation, which survive until E10.5, exhibit apoptosis at day E9.5 (Hohenstein, 2001).

In general, the time of embryonic arrest appears to reflect the region of the gene that is deleted. However, even careful characterization of growth arrest in embryos homozygous for the same knockout allele revealed that the time varied (Hakem, 1996; Liu, 1996; Ludwig, 1997). *Brca1*$^{-/-}$ blastocyst outgrowth in culture was generally poor (Liu, 1996; Ludwig, 1997; Shen, 1998), and these blastocysts were hypersensitive to γ-irradiation (Shen, 1998). Tumourigenesis studies of *Brca1*$^{+/-}$ mice on a *p53*$^{-/-}$ or *p53*$^{+/-}$ background revealed that even when mice were given whole-body γ-irradiation, tumour latency was not accelerated compared to similarly-treated mice lacking only *p53*. Tumours resulting in these γ-irradiated, *Brca1*$^{+/-}$, *p53*$^{+/-}$ or *Brca1*$^{+/-}$, *p53*$^{-/-}$ mice lacked both copies of *p53*, but generally retained a wildtype allele of *Brca1* (Cressman, 1999a; Cressman, 1999b; Hohenstein, 2001). Spectral karyotyping (SKY) analysis, in which each chromosome of a metaphase spread is "painted" with a different colour (Liyanage, 1996; Schrock, 1996b), of E9.5 MEFs from one null knockout (Table 1.3 #6) revealed that *Brca1*$^{-/-}$ MEFs had rearranged, abnormal karyotypes which were exacerbated on a *p53*$^{-/-}$ background (Shen, 1998). More details on the genomic changes in *Brca1*-associated tumourigenesis in the mouse will be discussed in section 1.9.5.

1.9.2 Double knockout models

To try and functionally rescue *Brca1*$^{-/-}$ embryos, several groups have crossed mice carrying *Brca1* knockout alleles onto other knockout backgrounds. While partial rescue of embryonic lethality is achieved on a *p53*$^{-/-}$ or *p21*$^{-/-}$ background, this rescue only extends embryonic development for one additional day (Hakem, 1997; Ludwig, 1997). The mouse knockout of *Bard1* is phenotypically very similar to that of *Brca1*, and the interdependence of these two genes is supported by the fact that

Brca1$^{-/-}$*, Bard1*$^{-/-}$ embryos are indistinguishable from *Brca1*$^{-/-}$ embryos (McCarthy, 2003). *Brca1*$^{-/-}$*, Brca2*$^{-/-}$ mice also have the same phenotype as *Brca1*$^{-/-}$ mice (Ludwig, 1997).

1.9.3 A humanized model of *Brca1*

Despite the fact that the overall identity of the mouse and human BRCA1 proteins is only 57%, human *BRCA1* is able to functionally rescue loss of *Brca1* in the mouse (Lane, 2000; Chandler, 2001). Chandler *et al.* generated transgenic mice carrying a bacterial artificial chromosome (BAC) which included the entire human *BRCA1* gene. These mice were mated to mice heterozygous for a *Brca1* knockout allele (Table 1.3 #11). Backcrossing to the *Brca1*$^{+/-}$ mice resulted in viable *Brca1*$^{-/-}$ mice which also carried the BAC. These mice were normal and healthy, and had no increased incidence of tumours up to 18 months, at time of publication (Chandler, 2001). This BAC transgenic model was modified slightly in a second paper, in which the human BAC used to rescue the *Brca1*$^{-/-}$ genotype carried a biologically relevant human mutation, introduced into the BAC by recombineering (Copeland, 2001), to generate a "humanized" mouse model. The mutation (T64G) mimics a common, cancer-related, RING-domain mutation, and was unable to rescue the *Brca1*$^{-/-}$ mice; *Brca1*$^{-/-}$ embryos with or without the BAC were indistinguishable. The "humanized" model was additionally used to determine that the mutation in the BAC-borne *BRCA1* gene causes aberrant splicing, resulting in premature termination of the BRCA1 protein. This BAC-rescue model demonstrates how such a "humanized" mouse model may provide valuable information about the molecular consequences of human mutations (Yang, 2003).

1.9.4 Alternative models suggest that loss of *Brca1* may not be sufficient for tumourigenesis

The goal of generating mouse models of *BRCA1*-related tumourigenesis was not met by the knockouts described above; thus, alternative alleles of *Brca1* were generated to try and develop such a model. Xu *et al.* generated a

conditional *Brca1* allele in which exon 11 was flanked by *loxP* sites (Table 1.3 #7). Cre-mediated deletion of this allele in ES cells generated a *Brca1* $\Delta X.11$ mutation. Despite the fact that the same group had previously generated an exon 11 knockout model which exhibited early embryonic lethality (Table 1.3 #6), mice homozygous for this newer, Cre-excised *Brca1* $\Delta X.11$ allele developed until E12.5-E18.5 (Shen, 1998; Xu, 1999c). The explanation for the difference in onset of growth arrest was that a *Neomycin phosphotransferase* (*Neo*) cassette had been left in intron 10 in the previous knockout, and this resulted in premature truncation of all *Brca1* transcripts (Brodie and Deng, 2001). *Brca1*$^{\Delta X.11/\Delta X.11}$ embryos still arrested but MEFs could be generated more easily from the older embryos. *Brca1*$^{\Delta X.11/\Delta X.11}$ MEFs were hypersensitive to γ-irradiation, lacked a G2-M checkpoint, and 25% of them had more than two centrosomes (Xu, 1999c). This indication of a potential role for BRCA1 in centrosome biology is supported by more recent evidence suggesting that BRCA1 interacts with α, β, and γ-tubulin, and co-localizes with tubulin at the centrosomes. Exon 11 appears to be important for this interaction, which is consistent with the phenotype of the Brca1$^{\Delta X.11/\Delta X.11}$ MEFs (Hsu and White, 1998; Deng, 2002; Lotti, 2002). More recent studies using this allele have demonstrated that *Brca1*$^{\Delta X.11/\Delta X.11}$ mice on a *p53*$^{+/-}$ background are viable, although they are prone to thymic lymphomas early in life (18 of 66 mice died by 28 weeks of age). Lymphoma formation appears to depend on the *p53* mutation, as all tumours investigated had lost the wildtype *p53* allele. *Brca1*$^{\Delta X.11/\Delta X.11}$ mice on a *p53*$^{-/-}$ background also developed thymic lymphomas (14 of 14 by ~15 weeks of age); this is more quickly than would be expected in *p53*$^{-/-}$ mice (additionally, *p53*$^{-/-}$ mice develop other types of tumour (Donehower, 1992)). This appears to indicate a role for mutated *Brca1* as an accelerant for *p53*-related tumourigenesis (Xu, 2001b; Bachelier, 2003).

The *Brca1*$^{\Delta X.11}$ conditional allele (*co*) was also used to generate conditional *Brca1* mice (one null allele and one conditional $\Delta X.11$ allele: *Brca1*$^{-/co}$), which were crossed to mice carrying a Cre transgene driven by one of two breast-specific promoters (Table 1.3 #8). These promoters were from whey acidic protein (WAP), a milk protein expressed in mammary epithelium during

pregnancy and lactation (Piletz and Ganschow, 1981; Robinson, 1995), and the MMTV-LTR (mouse mammary tumour virus LTR), which has been shown not to be breast-specific, but is expressed in breast epithelium and ductal cells (Wagner, 1997). Conditional mice carrying a Cre transgene showed developmental abnormalities of the mammary gland, but only a few breast tumours were observed in these animals after a long latency. Complete loss of *Brca1* did not appear to have occurred in most tumours. The addition of a $p53^{+/-}$ or $p53^{-/-}$ background to the conditional system accelerated tumourigenesis, but while tumours lacked both copies of *p53* and had other genetic alterations (described further in section 1.9.5), most retained the $Brca1^{co}$ allele, still functioning as a wildtype allele (Xu, 1999b), indicating that the loss of *Brca1* alone was not sufficient for tumourigenesis (Brodie, 2001; Weaver, 2002).

The same $Brca1^{co/-}$ conditional mice have also been used in conjunction with a Cre transgene driven by the bovine *keratin 5* (*K5*) promoter (Berton, 2003). In mice, this promoter is active in epithelial tissues of the oral and sinus cavities, esophagus, bladder, prostate, and vagina, as well as in the basal layer of the epidermis (Ramirez, 1994). Berton *et al.* observed that 72% (13 of 18) *K5*-Cre, $Brca1^{co/-}$ mice developed tumours by ~22 months of age, mainly in the inner ear canal or oral cavity. In a smaller study, tumourigenesis was accelerated by overexpression of *E2F1*, although the resulting tumours mainly occurred in the epidermis (Berton, 2003). The reasons for the effect of *E2F1* were not fully explored, but the link between E2F1 and BRCA1 was described earlier, in section 1.5.4. *Brca1* is overexpressed in *K5-E2F1* transgenic mice, likely due to the E2F1-responsive site in the *Brca1* promoter (Wang, 2000a; Berton, 2003). However, with or without *E2F1* overexpression, none of the mice developed mammary tumours, and tumourigenesis occurred only after a long latency.

Ludwig *et al.* generated a *Brca1* truncation mutation which mimics a mutation observed in human *BRCA1*-related breast tumours (Table 1.3 #9). Mice homozygous for this mutation were viable, but on a mixed 129Sv and C57BL/6 background, only 4% of the mice recovered from a heterozygous

intercross were homozygous for the mutation (Ludwig, 2001). Backcrossing to 129Sv mice, or outcrossing to the MF1 strain corrected the percentage to the expected 25%, although homozygous mutant males were infertile. Homozygous animals developed a variety of tumours with a mean latency of 17 months. While some animals did develop breast tumours, a wide variety of other tumour types were also observed. All tumours appeared to have secondary mutations as demonstrated by a change in gene product levels. This, and the long tumour latency, strongly suggested that while loss of *Brca1* could contribute to tumourigenesis, it was not in itself sufficient for tumourigenesis. Further, the variety of tumours suggested that Brca1 may not be a tissue-specific tumour suppressor in the mouse (Ludwig, 2001).

Jonkers *et al.* utilized mice co-conditional for both *Brca1* (exons 9-13 flanked by *loxP* sites) and *p53* (exons 2-10 flanked by *loxP* sites) (*Brca1*$^{c/c}$*, p53* $^{c/c}$ – see also Table 1.3 #10), which also carried a Cre transgene driven by the *Keratin-14* promoter (*K14*-Cre), active in skin and breast epithelia (Jonkers and Berns, 2003). Normally, tumourigenesis studies using a *p53*$^{-/-}$ background are compromised by the tumours these mice develop at an early age; in this study the *p53* mutation was conditional to reduce this problem. Of the 11 tumours analyzed from *K14*-Cre*, p53*$^{c/c}$, *Brca1*$^{c/c}$ mice, all had undergone recombination of both copies of *p53* and both copies of *Brca1*. In *K14*-Cre*, p53*$^{c/c}$, *Brca1*$^{+/c}$ mice, median tumour latency was roughly two times longer (330 days, versus 180 days), but of the 8 tumours analyzed, all had lost both copies of *p53* and retained at least one wildtype copy of *Brca1*. Breast and skin tumours developed in equal numbers (Jonkers and Berns, 2003). The conclusions were similar to those of the previous studies: *Brca1* loss was neither necessary nor sufficient for tumourigenesis, and the tissue-specificity of human *BRCA1*-related cancer was not mimicked.

1.9.5 Additional alterations in *Brca1*-related mouse tumours

To date, only a small number of murine *Brca1*-related breast tumours have been generated, and only limited data detailing the genetic changes in these tumours has been published. However, two groups have reported on the

presence or absence of a limited number of protein products in the mammary tumours from two different mouse models described above: the *Brca1*$^{co/-}$ mouse model, and the exon 11 truncation model (Table 1.3 #8 and 9) (Brodie, 2001; Ludwig, 2001; Weaver, 2002). In the former, a few of the tumours analyzed were from mice which also had a *p53*$^{+/-}$ background. Table 1.4 profiles some of the findings from these analyses. It is interesting to note that for some factors, such as the loss of the oestrogen and progesterone receptors, the mouse models mimic the characteristics of human *BRCA1*-related tumours. However, for some genes, such as Cyclin D1, the protein product is generally lost in human *BRCA1*-related tumours but not in murine ones. For other proteins such as ErbB2, the two mouse models did not agree, perhaps because the mice carry different alleles (Johannsson, 1997; Armes, 1999; Brodie, 2001; Ludwig, 2001; Lakhani, 2002; Weaver, 2002). The level of aneuploidy is generally higher in human *BRCA1*-related breast tumours compared to sporadic breast tumours (Johannsson, 1997). While structural and genomic abnormalities clearly occur in *Brca1*-related mouse tumours, no publication has related the amount of aneuploidy to that of sporadic control tumours (Xu, 1999b; Weaver, 2002).

These studies demonstrate that, whatever the role of *BRCA1* in human cancer, loss of *Brca1* in the mouse is not in itself sufficient for tumourigenesis. Moreover, the tissue specificity of human tumours is not fully recapitulated in the mouse. The reason for this difference (or the reason for the tissue specificity of *BRCA1*-related human tumours) is not clear. However, the choice of promoter for Cre expression in the conditional mouse models may be in part responsible. Arguments have been made against using milk-protein promoters for expression of breast-cancer genes, as these promoters are hormonally regulated, so would likely not be expressed in mammary stem cells. They also tend not to be expressed in ductal cells (a site of normal *Brca1* expression), which are thought to be a common site of tumourigenesis (Marquis, 1995; Rijnkels and Rosen, 2001). On the other hand, more widely-expressed (or ubiquitous) promoters may result in unwanted effects on other tissues. Promoters specific to mammary stem cells would be ideal, but none have yet been identified (Rijnkels and Rosen, 2001; Medina, 2002).

Table 1.4: Characteristics of mammary tumours in *Brca1* knockout mice, compared to human *BRCA1*-related tumours.

Models used are described in Table 1.3 (ΔX.11 conditional model, see Table 1.3 #8; tumours here were from *Brca1*$^{c/c}$; MMTV-Cre (n=6) or *Brca1*$^{c/c}$; MMTV-Cre; *p53*$^{+/-}$ (n=3) animals; *Brca1* truncation model, see Table 1.3 #9; all tumours from *Brca1*$^{tr/tr}$ animals).

Protein/characteristic	ΔX.11 *Brca1* conditional knockout model (n=9)	*Brca1* exon 11 truncation model (n=10)	Similar to human *BRCA1*-related tumours?	References
Loss of oestrogen receptor (ER-α) expression	7	5 (+4 downregulated)	Yes	(Armes, 1999; Brodie, 2001; Ludwig, 2001; Lakhani, 2002)
Loss of progesterone receptor (PR) expression	Not measured	7	Yes	(Armes, 1999; Ludwig, 2001; Lakhani, 2002)
Loss of ErbB2/neu expression	2	6 (+1 downregulated)	Mouse models not consistent; human tumours generally have lost expression.	(Armes, 1999; Brodie, 2001; Ludwig, 2001; Lakhani, 2002)
Expression of Cyclin D1 detected	9	10	No	(Armes, 1999; Brodie, 2001; Ludwig, 2001)

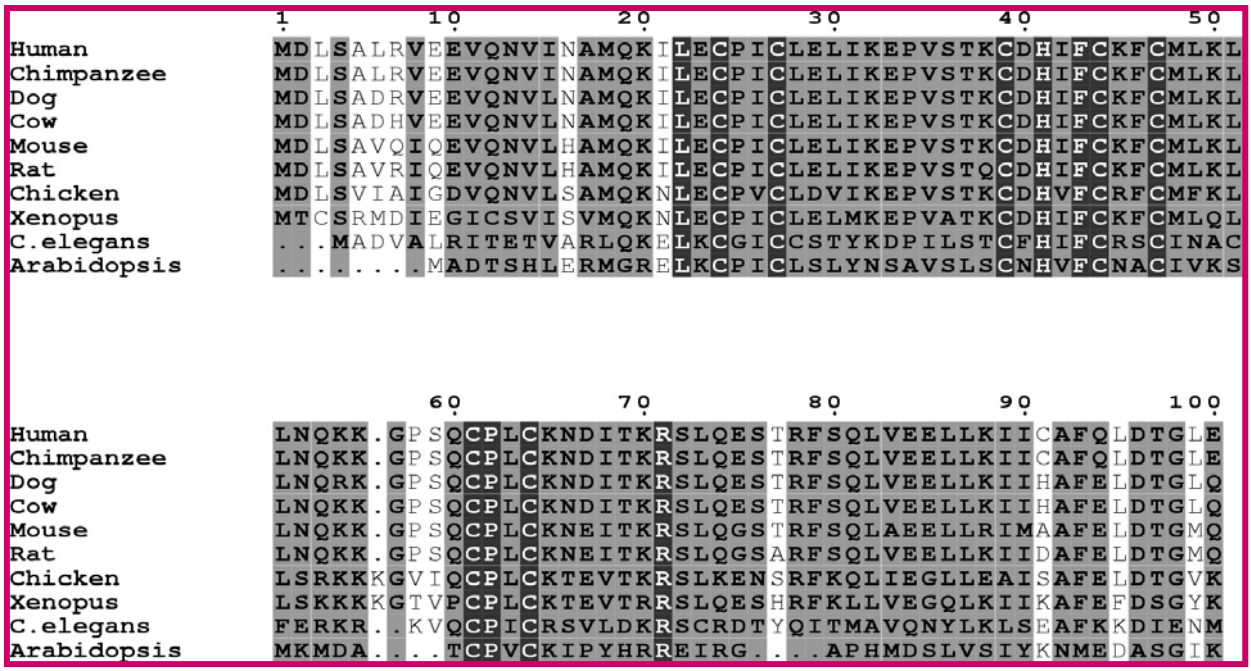
However, while *BRCA1*-related breast cancer may be difficult to model using knockout alleles of *Brca1* in mice, the underlying mechanisms for tumourigenesis – *i.e*., the caretaker roles of BRCA1 in cell cycle control and the response to DNA damage – can be very adequately investigated using these mice. In fact, it is here that the underlying similarity between not only the mouse and human, but also more evolutionarily distant homologues, is most compelling.

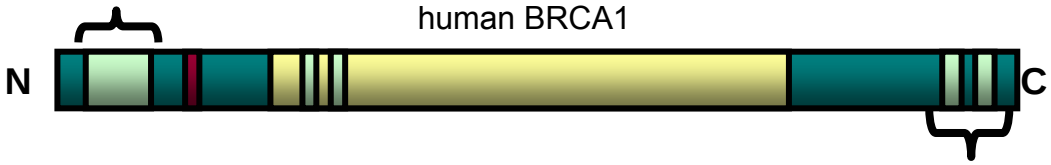
1.10 NON-MAMMALIAN HOMOLOGUES OF *BRCA1*

Homologues of human *BRCA1* were first thought to exist only in other mammals, but *BRCA1* homologues have now been identified in chicken (Orelli, 2001), *Xenopus* (frog) (Joukov, 2001b), *C. elegans* (Boulton, 2004), *Arabidopsis thaliana*, and *Oryza sativa* (rice) (Lafarge and Montane, 2003). All these homologues were identified by their well-conserved RING domain and BRCT repeats (Figure 1.11). In fact, in *C. elegans* or the plants, these domains comprise the majority of the protein (Lafarge and Montane, 2003; Boulton, 2004). While the overall similarity between the *Xenopus* or *C. elegans* homologues and the human BRCA1 protein is not as high as that of the mouse-human similarity, an alignment of the RING domains from several homologues from human to *Arabidopsis* shows that the key C and H residues of the RING motif are absolutely conserved (Figure 1.11). A *BARD1* homologue has also been identified in *Xenopus*, chicken, and *C. elegans* (Joukov, 2001b; Orelli, 2001; Boulton, 2004).

The conservation of BRCA1-related phenotypes amongst the different species is striking. In *C. elegans*, RNA-interference (RNAi, which decreases the expression of the target gene) of *BRC-1* or *BRD-1* (the *C. elegans BRCA1* and *BARD1* homologues) results in cell-cycle checkpoint–independent apoptosis, which is increased in response to γ-irradiation. BRC-1 also forms damage-induced nuclear foci (Boulton, 2004). Antisense-mediated depletion of x*BRCA1* or x*BARD1*, the *Xenopus* homologues of *BRCA1 and BARD1*, results in severe developmental defects in later-stage embryos, non-viable frogs, and higher levels of aneuploidy in cells (Joukov, 2001b). *Arabidopsis*

a.





b.

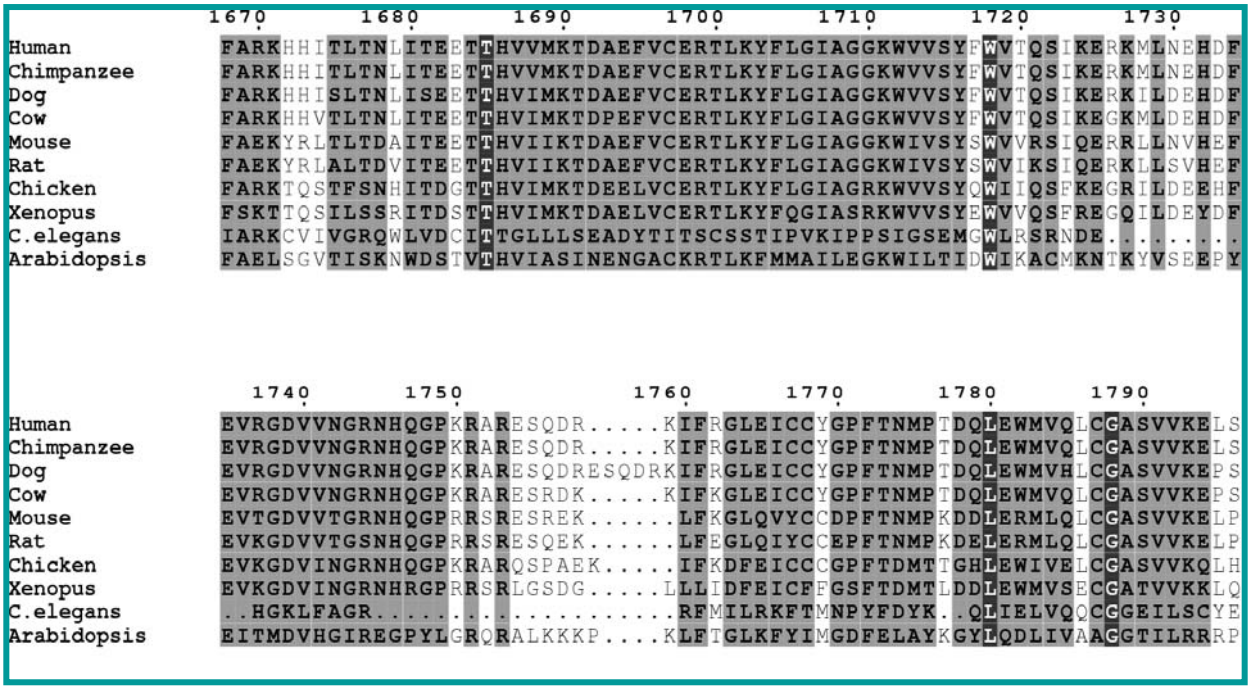


Figure 1.11: Multiple alignments showing conservation of the RING domain and BRCT repeats. a. RING-domain alignment for indicated species. Identical residues are in dark grey, similar ones in light grey. Note that the key cysteine (C) and histidine (H) residues of the zinc-finger are absolutely conserved. **b.** BRCT repeats, aligned as above.
ClustalW and ESPript (Blosum62 matrix) were used to produce alignments.

thaliana BRCA1 is upregulated in plantlets in response to γ-irradiation and appears to be upregulated at the same time as the *Arabidopsis Rad51* gene (Lafarge and Montane, 2003). On the whole, these phenotypes are strongly reminiscent of those seen in *Brca1* knockout mice or in *BRCA1*-deficient mouse or human cell lines.

1.11 DNA REPAIR

1.11.1 Focal indication of a role for BRCA1 in the response to DNA damage

BRCA1 clearly plays roles in various types of DNA damage repair. Following exposure to γ-irradiation (which mainly causes double-stranded breaks), MMC (a DNA cross-linking agent), UV light, or a HU-induced DNA replication block, normal S phase BRCA1 foci disappear (Scully, 1997b; Zhong, 1999; Wu, 2000). BRCA1 reappears in foci later as soon as an hour after damage, and these damage-induced foci may persist for 8-12 hours post-damage.

Damage-induced foci do not have the same composition as BRCA1 S phase foci; after damage, BRCA1 is hyperphosphorylated on various residues and the foci appear to include a different subset of proteins (Scully, 1997b; Wang, 2000b). While proteins like RAD51, BRCA2, and BARD1 localize with BRCA1 in both S phase and damage-induced foci, PCNA (proliferating cell nuclear antigen), which forms BRCA1-independent foci during S phase, co-localizes with BRCA1 following damage, presumably at replication forks (Scully, 1997b; Scully, 1997c; Chen, 1998; Wang, 2000b). Other proteins which co-localize with BRCA1 following DNA damage include the RAD50-MRE11-NBS1 complex involved in DSBR, the histone protein H2AX (Celeste, 2002), FANCD2 (D'Andrea and Grompe, 2003), BLM, and other proteins which comprise the BASC (Wang, 2000b). In mouse $Brca1^{\Delta X.11/\Delta X.11}$ MEFs, which lack the Rad51-interaction domain coded by exon 11, Rad51 foci do not form following γ-irradiation (Huber, 2001). However, the mutant form of *BRCA1* in the human cancer cell line HCC1937 does carry the RAD51-interaction domain, and RAD51 foci form normally following γ-irradiation (Zhong, 1999).

RAD50 foci also appear to form normally in cells lacking BRCA1 (Wang, 2000b; Wu, 2000).

1.11.2 Damage-induced phosphorylation of BRCA1

BRCA1 is phosphorylated normally during certain parts of the cell cycle, but phosphorylation also occurs following DNA damage. Some studies have indicated that damage-induced phosphorylation may depend on factors such as cell cycle stage and the dose of damage received (Scully, 1997b; Okada and Ouchi, 2003). Several kinases may phosphorylate BRCA1 in response to DNA damage, the two key ones being ATM (Ataxia telangiectasia mutated) and ATR (ATM and RAD3-related), both members of the phosphatidylinositol 3-kinase (PI3K) family. These two kinases phosphorylate many cell-cycle proteins and are important both in normal checkpoint control and the response to DNA damage (Shiloh, 2001). In humans, the recessive disorder ataxia-telangiectasia (AT) results from a loss of *ATM*, and is characterized by neuronal degeneration (cerebellar ataxia), sterility, and a greatly increased cancer risk. Cell lines from AT patients show chromosomal breakage and telomere instability and are hypersensitive to ionizing radiation (but not to base-damaging agents such as UV light) (reviewed in Thompson and Schild, 2002). No human disorder has been attributed to mutations in the *ATR* gene. The corresponding mouse knockout models mimic the human conditions fairly well; homozygous *Atm* knockout mice are infertile, hypersensitive to ionizing radiation, and succumb early in life to thymic lymphomas (Barlow, 1996; Xu, 1996), while homozygous *Atr* knockout embryos die early in embryonic development, before E7.5 (Brown and Baltimore, 2000).

Evidence that ATM and ATR phosphorylate BRCA1 has come from a number of experiments, including investigating the phosphorylation status of endogenous BRCA1 before and after DNA damage in AT cells or in cells constitutively expressing a dominant-negative form of ATR (Cortez, 1999; Gatei, 2001). Phosphorylation of overexpressed, tagged BRCA1 protein in such cells has also been monitored using phosphorylation-specific BRCA1 antibodies; these studies helped to define the residues targeted by each

kinase (Tibbetts, 2000; Gatei, 2001). The roles of ATM and ATR are not absolutely delineated, but the current understanding is that ATM appears to phosphorylate BRCA1 following γ-irradiation, but not after UV exposure (Cortez, 1999; Gatei, 2000; Tibbetts, 2000). ATR phosphorylates BRCA1 following UV exposure, and also may phosphorylate BRCA1 to some extent following γ-irradiation (Tibbetts, 2000; Gatei, 2001). The actual mechanisms are more complex than this summary indicates, as kinases downstream from ATM, or possibly independent kinases may also phosphorylate BRCA1 (Ruffner and Verma, 1997; Altiok, 1999; Lee, 2000; Foray, 2002). However, the PI3K kinase DNA-PKcs (DNA protein kinase, catalytic subunit), which plays a major role in both non-homologous end joining (NHEJ) and V(D)J (variable(diverse)joining) recombination of T- and B-cell receptor genes, does not appear to phosphorylate BRCA1 following DNA damage (Scully, 1997b).

Both ATM and ATR may have other links with BRCA1; ATM and BRCA1 have been shown to interact by co-immunoprecipitation (ATM is part of the BASC), and co-localize to some damage-induced nuclear foci (Cortez, 1999; Tibbetts, 2000; Wang, 2000b). In addition, BRCA1 may be necessary for a certain subset of ATM and ATR phosphorylation activities, as abnormal phosphorylation of targets of these kinases in HCC1937 cells, which lack wildtype BRCA1, is observed. Phosphorylation of some of these targets returns to normal upon expression of wildtype BRCA1 (Foray, 2003).

The clearest conserved phenotype of BRCA1 and its homologues is a role in the response to DNA damage. Since DNA damage can be broadly classified into double-strand breaks and damage to bases (Figure 1.12), these two categories will be considered separately. Evidence indicates that BRCA1 plays roles in repairing both types of damage.

1.11.3 Double-strand break repair

1.11.3.1 An overview of DNA double-strand break repair

DNA double-strand breaks (DSBs) may be caused such agents as γ-irradiation, free radical attack, or strand crosslinks. These lesions are

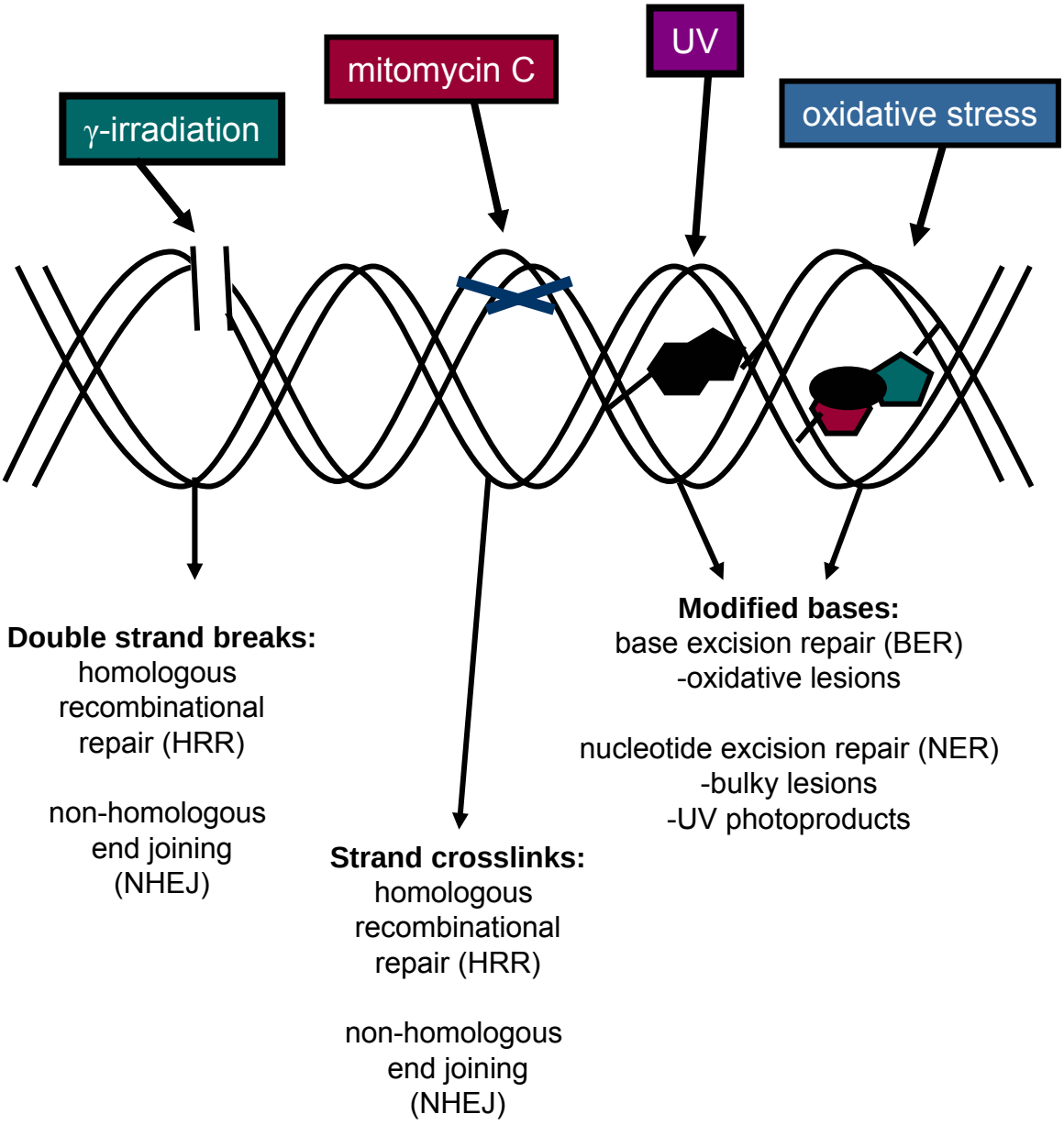


Figure 1.12: Representative types of DNA damage and primary repair pathways for each. Schematic showing common mutagens used in damage-repair research, their characteristic lesions, and the pathways used to repair them.
UV=ultraviolet light irradiation.

repaired by two major pathways: homologous recombinational repair (HRR) and non-homologous end joining (NHEJ) (Figure 1.13). HRR utilizes a homologous chromosome or sister chromatid to precisely repair the chromosome, while NHEJ simply rejoins the break in a sequence-independent manner. HRR is a high-fidelity repair process, while NHEJ often is not; exonuclease activity at break ends can cause a loss of genetic information, and the mechanism of NHEJ itself generally results in a small insertion or deletion at the break site. Many of the proteins which play key roles in detection and repair of strand breaks were first discovered through yeast screens for repair-deficient strains, and a large number of these proteins have homologues in higher eukaryotic organisms (reviewed in Chu, 1997; Thompson and Schild, 2001).

1.11.3.1.1 The balance between NHEJ and HRR

Mammalian cells were once thought to primarily repair DSBs through NHEJ, but recent evidence suggests that HRR is involved in repairing at least 30-50% of DSBs in mammals (Liang, 1998). The impetus to use HRR or NHEJ for repair appears to depend on several factors, including the stage of the cell cycle. Studies using *Rad54*$^{-/-}$ (HRR deficient) or *Ku70*$^{-/-}$ (NHEJ deficient) chicken DT40 cells showed that *Rad54*$^{-/-}$ cells are γ-irradiation sensitive in late S and G2 phases, while *Ku70*$^{-/-}$ cells are γ-irradiation sensitive in G1 and early S phases. Cells lacking both proteins are more sensitive to γ-irradiation than the single mutants, which suggests that these pathways do not fully complement one another (Takata, 1998; Wang, 2001b). This may be due to a dependence on the availability of an appropriate substrate for HRR, as the use of HRR in G1 would result in LOH of the repaired area. This is supported by evidence showing that in mouse ES cells, a sister chromatid is used more often than a homologue for HRR (Johnson and Jasin, 2000).

Kinetically, double-strand break repair (DSBR) occurs in a biphasic manner – there is a “fast” component of repair over the first 30-60 minutes which mends some 80% of breaks, and a “slow” phase which works on the remaining breaks over the next 24 hours (Figure 1.14). Repair of genomic DNA is generally monitored using pulsed-field gel electrophoresis, which allows

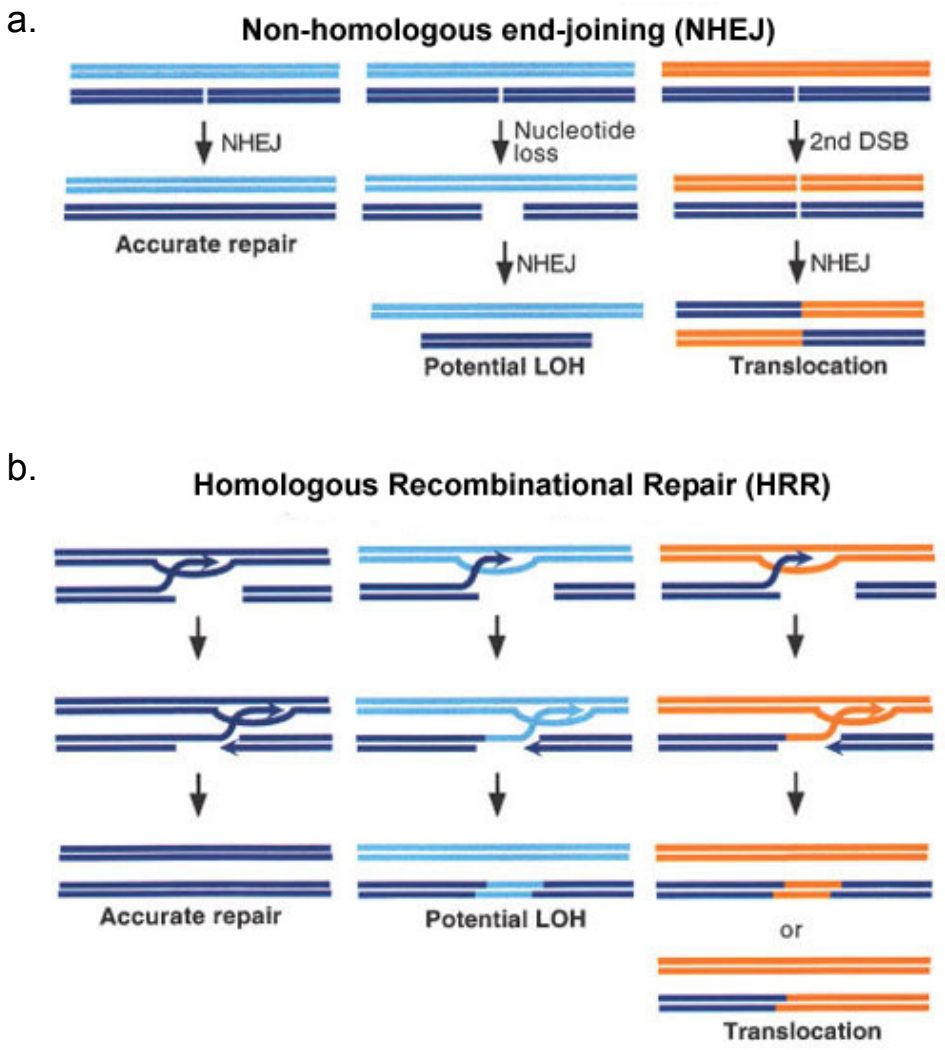


Figure 1.13: Non-homologous end joining (NHEJ) and Homologous Recombinational Repair (HRR) of double-strand breaks. a. NHEJ rejoins a double-strand break in a sequence-independent manner. Three possible outcomes, including loss of genetic information, are shown. **b.** HRR uses a homologous chromosome or sister chromatid to accurately repair a double-strand break. Depending on the substrate used for repair, loss of heterozygosity (LOH) may occur, but generally a net loss of genetic information does not occur. Figure modified from (Ferguson and Alt. Oncogene 20:5572, 2001.

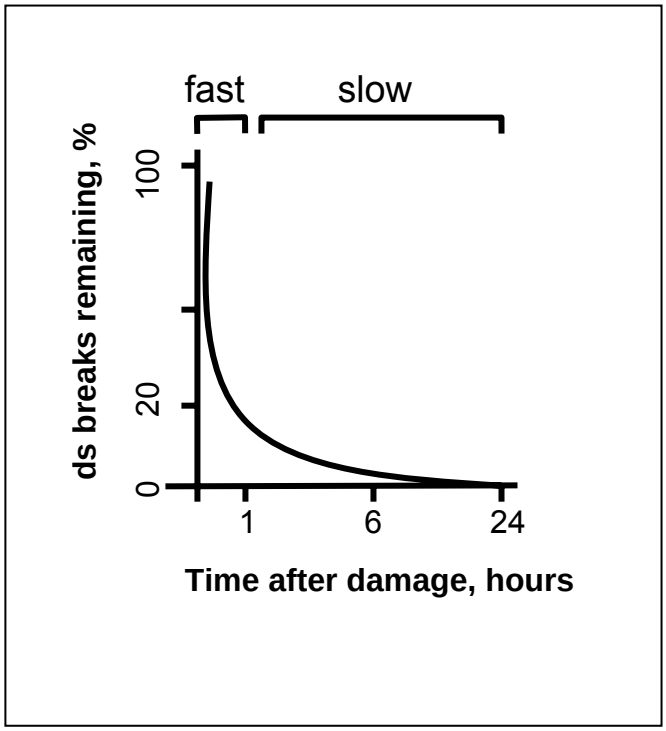


Figure 1.14: **Double-strand break repair kinetics.**
Typical double-strand break repair kinetics showing “fast” repair over the first hour post-damage and “slow” repair following.
ds: double-strand.

separation of very large pieces of DNA; repaired DNA runs more slowly than broken DNA, allowing the percentage of faster-running, broken DNA to be measured over time. Generally, even the "slow" phase is virtually complete by six hours post-damage. Most evidence indicates that "fast" repair is done by NHEJ and the "slow" repair by HRR (reviewed in Biedermann, 1991; Iliakis, 1991; DiBiase, 2000). For example, cells lacking DNA-PKcs (a key protein in NHEJ), are hypersensitive to γ-irradiation. Their "fast" and "slow" repair kinetics do not change, but a much smaller proportion of breaks are repaired by the "fast," or NHEJ, component of repair. The γ-irradiation hypersensitivity of these cells is hypothesized to result from the increased fraction of breaks left for the "slow" component to repair, despite the fact that the "slow" component is working normally (Iliakis, 1991; DiBiase, 2000). These kinetics may help explain the presence of BRCA1 foci in normal cells after most DNA repair has already taken place. While most DSBs are mended in the first couple of hours following DNA damage, the "slow" component of repair may continue to work on breaks for some hours past that time.

1.11.3.1.2 Early Cellular Responses to DSBs

The cellular response to double-strand breaks begins with recognition or detection of lesions, followed by the triggering of downstream repair/reaction events. The kinases ATM and ATR (discussed in section 1.11.2) appear to be key players in the recognition of strand breaks; they phosphorylate and activate an overlapping but distinct set of targets, triggering repair or other downstream processes in response to breaks (reviewed in Jackson, 2002). The global importance of ATM is underscored by the fact that human A-T cells are deficient in ionizing-radiation-induced G1-S, intra-S, and G2-M phase checkpoints (Lavin and Shiloh, 1997). ATM is activated minutes after damage, and can itself bind DNA ends (reviewed in Thompson and Schild, 2001). Other damage-recognition factors may also exist.

Other early events in repair are likely to include changes in histone proteins to help relax chromatin structure around the break to allow for access by repair proteins. In mammals, this includes the phosphorylation of the histone protein H2AX, an early event following exposure to damaging agents (Rogakou,

1998). A an increase in cellular deoxribonucleotides used for repair-related synthesis is also likely to occur (Tanaka, 2000).

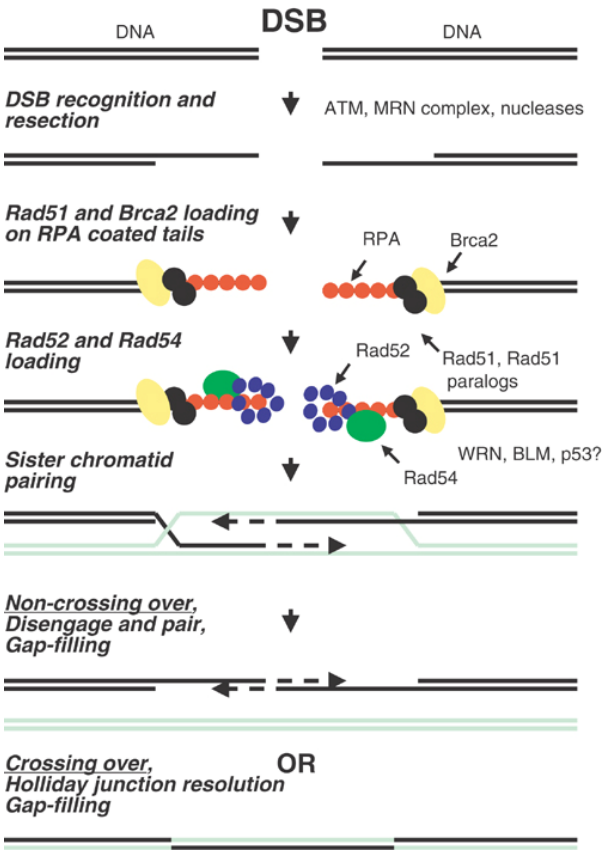
In addition to triggering repair-related pathways in response to DNA damage, the ATM and ATR kinases may also induce cell-cycle arrest or delay (the key cell-cycle checkpoint proteins CHK1 and CHK2 are phosphorylated and activated by ATR and ATM, respectively) to allow additional time for repair. If the damage is overwhelming an apoptotic pathway may be triggered, possibly by ATM and/or ATR: both directly phosphorylate the p53 protein, and ATM may also indirectly induce accumulation of p73 to induce apoptosis (reviewed in Dasika, 1999; Bernstein, 2002; Thompson and Schild, 2002; Iliakis, 2003).

1.11.3.1.3 Proteins Involved in Homologous Recombinational Repair

Homologous recombination repair is thought to begin with resection of the DSB into a single-stranded 3' overhang which invades a double-stranded homologous region (see Figure 1.15a for an overview of the process). Resection may involve the mammalian RAD50-MRE11-NBS1 complex - RAD50 is thought to bind DNA, and the complex has both exo- and endo-nuclease activities *in vitro*, as well as helicase activity. However, the MRE11 nuclease tends to generate 5' overhangs, leading some to suggest that this complex may play more of a an "organizer" role in break repair (reviewed in Thompson and Schild, 2001).

A key protein in HRR is RAD51, a mammalian orthologue of the bacterial RecA protein. RAD51 forms a filament on ssDNA overhangs, and its ability to hydrolyze ATP appears to be necessary for recombination (Thompson and Schild, 2001; Jackson, 2002; Thompson and Schild, 2002). Initially, the ssDNA region is likely to be coated with the heterotrimeric Replication Protein A (RPA), a protein with high affinity for ssDNA which is involved in replication, DNA repair, and recombination (Wold, 1997). Binding of RPA to the ssDNA region is important for the formation of an even coating of RAD51 along the ssDNA, as the helix-destabilizing properties of RPA minimize secondary structure, especially when the region of ssDNA is long (Treuner, 1996; Sung, 2003). Since RPA has a high affinity for ssDNA, RAD51 alone displaces RPA

a.



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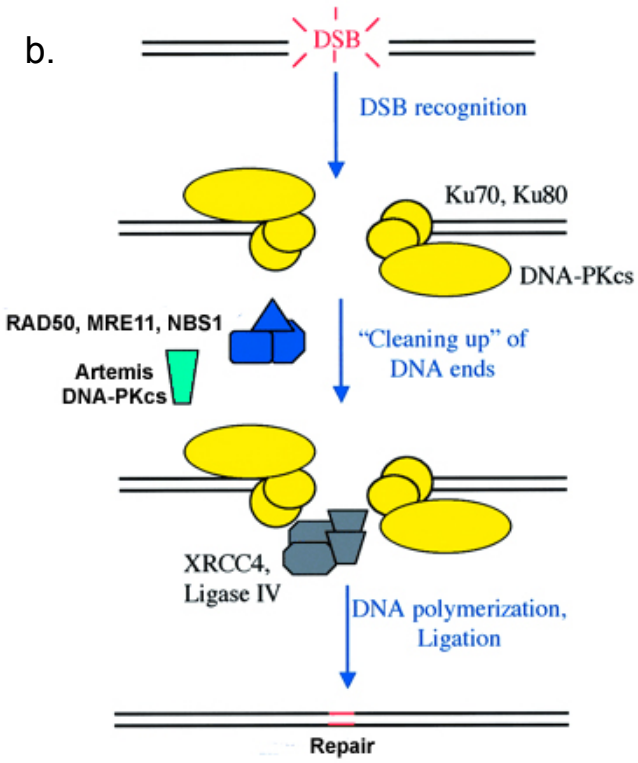


Figure 1.15: Proteins and mechanisms involved in HRR and NHEJ double-strand break repair. a. Homologous recombinational repair, diagram showing key proteins and mechanisms involved. Note the dual possible outcomes of the Holiday junction following strand invasion. Figure taken from (Valerie, 2003).
b. Proteins and mechanism (simplified) of NHEJ, showing recognition of the break site, binding of the DSB end by the Ku proteins, end modification, and religation of the break site. Figure modified from (Jackson, 2002).

very slowly. RAD52 interacts with both RPA and RAD51, and greatly accelerates the displacement of RPA by RAD51 (Sugiyama and Kowalczykowski, 2002; Symington, 2002; Kantake, 2003), although BRCA2 (which also interacts directly with RAD51) also appears to aid in loading or organization of RAD51 on ssDNA (Yang, 2002). RAD52 is thought to be one of the key factors in the "decision" to repair a break by HRR or NHEJ, as it forms a multimeric complex which will bind to double-stranded ends, and may compete with the NHEJ-related Ku heterodimer to bind free DNA ends (reviewed in Symington, 2002). Loss of RAD52 in yeast results in severe HRR defects, although loss of Rad52 in mice is not a lethal event (unlike loss of Rad51) (Lim and Hasty, 1996; Rijkers, 1998).

Once the RAD51-ssDNA nucleoprotein filament is formed and a homologous dsDNA section has been identified, strand invasion occurs, with displacement of one strand appearing as a D-loop. RAD52 and RAD54 assist in the invasion of the RAD51-coated ssDNA into a homologous section of dsDNA; RAD54 may increase the efficiency of ss/ds DNA pairing and appears to have helicase activity. A DNA polymerase extends the 3' terminus of the invading strand, copying the information from the homologous partner, and the break is ligated by DNA ligase I (Thompson and Schild, 2001). Migration and resolvation of the Holiday junction may then occur, although, in mammalian cells, an alternative non-crossover pathway may predominate in which the Holiday junction disengages (illustrated in Figure 1.15a). This mechanism would protect against LOH at the region, but further experimental work is needed to define how frequently this pathway is used and if novel proteins are involved in this mechanism (Johnson and Jasin, 2000).

The mechanism of HRR is more complex than this summary indicates. In addition to RAD51, there are several mammalian RAD51 paralogues (RAD51B, C, and D, XRCC2, and XRCC3) which appear to be involved in strand invasion and junction resolvation, as well as a number of other proteins which are likely to be involved either in regulation or mechanism (reviewed in Thompson and Schild, 2001; Thompson and Schild, 2002).

1.11.3.1.4 Proteins Involved in Non-homologous end-joining

Several of the core proteins involved in mammalian NHEJ also mediate V(D)J recombination in immune cells; the results of a protein deficiency in one of these proteins often leads to severe immunodeficiency disorders as well as problems with repair of DSBs (Lieber, 2003).

Key elements in NHEJ include the Ku heterodimer (Ku70/Ku80), which forms a ring-type structure and threads onto free DNA ends (see Figure 1.15b for an overview of the NHEJ process). As mentioned above, the Ku proteins may compete with RAD52 to bind to free ends. The Ku heterodimer is likely to be the localization signal for DNA-PKcs, a key kinase in NHEJ and in V(D)J recombination which is appears to be activated by the presence of DNA breaks. The importance of this protein is underscored by the fact that the scid (severe combined immunodeficiency) mutation in mice and humans results from lack of DNA-PKcs (Hendrickson, 1991; Thompson and Schild, 2001). Once activated, the targets of DNA-PKcs are likely to include XRCC4, which interacts with and stimulates the activity of DNA ligase IV, responsible for ligation of the DSB (reviewed in Dasika, 1999).

As in HRR, processing of the break region generally occurs. Break sites which cannot be directly re-ligated are often sites for limited addition/deletion of bases. Additionally, NHEJ often occurs at an area of microhomology (1-4 identical bases). As a result of microhomology joining, gaps or overhangs are often left which must be filled in or removed (Lieber, 2003). The RAD50-MRE11-NBS1 complex may be responsible for NHEJ-related processing; not only does the this complex possesses exo- and endonuclease activity, but yeast strains which lack these proteins (Rad50, Mre11, or Xrs2 – the third yeast protein) are deficient in NHEJ (Jackson, 2002). However, a second processing complex in vertebrates has been more recently identified, composed of DNA-PKcs and the protein Artemis. Artemis mutations are found in a subset of human scid patients who have an increased sensitivity to ionizing radiation (Moshous, 2001). The DNA-PKcs/Artemis complex has endonucleolytic activity at 3’ and 5’ overhangs, as well as the ability to open hairpins (important for V(D)J recombination) (Ma, 2002; Lieber, 2003).

Despite their importance in mammalian cells, Artemis and DNA-PKcs have only been identified in vertebrates to date, in contrast to the Ku proteins, which have homologues in all eukaryotes examined (Jackson, 2002; Lieber, 2003). It is not clear at present whether this indicates that other kinases fill the role of DNA-PKcs in non-vertebrates. Scid mice are not only deficient in V(D)J recombination, they are hypersensitive to γ-irradiation, which argues that DNA-PKcs is necessary for NHEJ in vertebrates (Berton, 2003). It is possible that in non-vertebrates, the RAD50-MRE11-NBS1-type complex is responsible for end-processing of double-strand break ends prior to NHEJ, while in vertebrates, the DNA-PKcs/Artemis complex can fill this role in addition to its vital role in processing intermediates during V(D)J recombination (Lieber, 2003).

The actual process of HRR or NHEJ is more complex – and is likely to involve more complex interactions than these summaries indicate. Additionally, unanswered questions remain, including whether a polymerase is involved in NHEJ or not (Lieber, 2003). In recent years, the involvement of a large number of other proteins involved in the process or regulation of DSBR has been shown. A number of these proteins are implicated in human cancer syndromes, such as the Bloom's Syndrome helicase BLM, the Fanconi anemia FANC proteins, BRCA2, and BRCA1 (which will be discussed further in the next sections) (reviewed in Thompson and Schild, 2002). The study of these proteins in mice is compounded by the fact that mouse knockouts of the genes are often embryonic lethal (*Rad50, Rad51, Rad51b, Rad51d, Atr, Xrcc4, Brca1*, *Brca2*, and *DNA ligase IV*), precluding extensive studies of the effects of loss of the gene product on the whole organism (reviewed in Dasika, 1999; Thompson and Schild, 2001). Other knockouts, such as those of *Ku*, *Atm*, *Rad54,* or DNA-PKcs/scid, are viable, but the mice or cells are often hypersensitive to double-strand break-inducing agents, have immune defects, or, in the case of Ku knockout mice, show signs of premature aging (Hendrickson, 1991; Barlow, 1996; Xu, 1996; Thompson and Schild, 2001; Jackson, 2002).

1.11.3.2 BRCA1 and Homologous Recombinational Repair (HRR)

Evidence that BRCA1 is involved in HRR of DSBs comes from several sources, including its interactions with proteins involved in HRR, the altered repair kinetics of cells lacking BRCA1, and a decreased ability of *Brca1*$^{-/-}$ mouse ES cells to successfully integrate a targeting cassette or repair a break via homologous recombination.

As described earlier, BRCA1 interacts with numerous proteins implicated in homologous recombination, including RAD51 (Scully, 1997c), ATM (Cortez, 1999; Gatei, 2000; Tibbetts, 2000), BLM (Wang, 2000b), and two components of the RAD50-MRE11-NBS1 complex: the Nijmegen breakage syndrome protein NBS1, and RAD50 (Varon, 1998; Wang, 2000b).

Several groups have examined the response of the HCC1937 human cell line (which carries only a C-terminal truncated version of BRCA1) to γ-irradiation (Table 1.5, experiments 1-3). In all three cases, "fast" repair of breaks during the first hour after damage was equally efficient in HCC1937 cells and controls (Abbott, 1999; Foray, 1999; Scully, 1999). Two groups showed that the remaining breaks were competently repaired only by the control cells; at either 6 or 24 hours post-damage, HCC1937 cells had not finished DSBR (Foray, 1999; Scully, 1999). However, a third group demonstrated that all the γ-irradiation-induced breaks in HCC1937 cells and controls were repaired by four hours post-damage. In this case, the majority of DSBs were repaired within the first two hours, which may have left too few breaks to allow an accurate measurement of the rate of the "slow" component (Abbott, 1999). HCC1937 cells were also hypersensitive to γ-irradiation when compared to controls (Abbott, 1999; Foray, 1999). These three studies indicate that cells lacking BRCA1 may have a defect in the "slow", or homologous recombinational, component of repair – albeit using a cell line which is known to have a number of mutations besides the one in *BRCA1* (Tomlinson, 1998).

The existence of a mouse *Brca1*$^{-/-}$ ES cell line provides a simple way of measuring homologous recombination efficiency, namely gene targeting. Two conventional gene-targeting vectors integrated correctly into *Brca1*$^{-/-}$ cells at a

Table 1.5: Repair response of HCC1937* cells to double-strand breaks.

#	Cell line(s) used	γ-irradiation dose	Repair, one hour after damage	Repair, six hours after damage	Repair, 24 hours after damage	γ-irradiation hypersensitivity?+	Reference
1	HCC1937+Ad*BRCA1-GFP* (adenovirally expressed at near-endogenous levels)	8 Gy	50%	Nearly complete	Complete	No, compared to HCC1937 control	(Scully, 1999)
	HCC1937+Ad*GFP* (empty adenoviral vector)		50%	~60%	~75%	Yes	
2	HCC1937	30 Gy	~50%		58%	Yes	(Foray, 1999)
	Lymphoblast cell line (wildtype)		~50%		Nearly complete	No	
3	HCC1937	10 Gy	Most (no numbers given)	No breaks observed after 4 hours		Yes	(Abbott, 1999)
	HCC1937 + *BRCA1* lacking aa 702-843 (this mutant does not cause growth suppression when overexpressed)		Most (no numbers given)	No breaks observed after 4 hours		No	
4	HCC1937	40 Gy	92%	98%	Complete	Not measured	(Wang, 2001)
	HCC1937 + 30 μM wortmannin (to inhibit NHEJ)		78%	83%	89%	Not measured	

* The HCC1937 human cell line carries only a C-terminal mutated version of BRCA1.
+ γ-irradiation hypersensitivity compared to control cell line shown for each experiment unless otherwise noted. Gy=gray (dose of γ-irradiation).

significantly lower rate than into wildtype cells (an estimated 13-fold reduction, once corrected for the 4-fold increase in random integration) (Moynahan, 1999). The use of a cassette containing an I-SceI site (cleavage by the nuclease SceI at an I-SceI site generates a DSB) designed to distinguish between HRR or NHEJ repair events revealed a 5- to 6-fold decrease in the amount of HRR, and a 1.5-fold increase in NHEJ (Moynahan, 1999). A third study using a similar I-SceI-containing reporter cassette again demonstrated a significant decrease in HRR efficiency in *Brca1*$^{-/-}$ cells (Moynahan, 2001). All of these experiments utilized ES cells homozygous for a *Brca1* Δ*X.11* allele (Table 1.3 #5) (Gowen, 1996).

Moynahan *et al.* also showed that the homologous recombination efficiency of a *Brca1*$^{+/-}$ ES cell line is identical to that of wildtype cells (Moynahan, 2001). Similar studies using human cancer cell lines demonstrated a heterozygous effect; that is, a heterozygous cell line showed an intermediate phenotype as compared to wildtype and *BRCA1*-deficient cell lines (Abbott, 1999; Foray, 1999; Baldeyron, 2002). A caveat must be added to these results: the *Brca1*$^{\Delta X.11/\Delta X.11}$ ES cell line used in these studies was the only double-targeted line ever recovered by this group (Gowen, 1996), and the addition of a *Brca1*-containing transgene could not fully rescue either the HRR defect or hypersensitivity to MMC. However, retargeting a wildtype allele back into the *Brca1* locus fully rescued hypersensitivity to MMS. This indicates that recovery of double-targeted ES cells is a rare event, but rescue of the phenotype by retargeting suggests that there is not a secondary mutation in this cell line which affects the *Brca1*-related phenotype (Moynahan, 2001).

Overall, these data from both human and mouse cell lines indicates that BRCA1 does indeed play a role in HRR.

1.11.3.3 BRCA1 and non-homologous end joining (NHEJ)

While a good deal of evidence argues that BRCA1 is involved in HRR, evidence related to its role in NHEJ is not as clear or consistent.

The HCC1937 cell line exhibited normal “fast” repair in all three studies described above (Table 1.5, experiments 1-3). Two additional studies using wortmannin, an inhibitor of the NHEJ-related DNA-PKcs kinase, further suggest that HCC1937 cells do not have an NHEJ defect. HCC1937 cell extracts were as efficient as control cell extracts in mediating end rejoining in an *in vitro* assay (Merel, 2002). Additionally, the repair kinetics of HCC1937 cells following γ-irradiation in the presence or absence of wortmannin showed that wortmannin inhibition of DNA-PKcs (and thus NHEJ) meant that cells repaired fewer breaks in the first hour (78% vs. 92%), but that “slow” repair occurred with unchanged kinetics (Table 1.5, experiment 4). DSBR was virtually complete by 24 hours, regardless of the presence of wortmannin, likely because the “fast” component of repair was not fully inhibited and still repaired the majority of breaks (Wang, 2001a). In summary, the experiments in Table 1.5 suggest that HCC1937 cells do not have a deficiency in NHEJ. Indeed, a recent experiment using the HCC1937 cell line indicates that there may be an increase in NHEJ in these cells (using an assay for random plasmid integration) which is restored to wildtype levels on expression of a *BRCA1* transgene, suggesting that BRCA1 may normally function in suppression of NHEJ in favor of HRR (Zhang, 2004).

NHEJ proteins are instrumental in V(D)J recombination of immune cells, as evidenced by the phenotype of scid mice, which lack the instrumental DNA-PKcs kinase involved in NHEJ and do not develop mature T or B cells (Blunt, 1995; Kirchgessner, 1995). V(D)J recombination in T and B cells is slightly different: the genes used to generate the T- or B-cell receptors differ, but the same set of proteins carry out the mechanics of recombination (Gellert, 2002). A conditional *Brca1* mouse was generated which carried one null and one *loxP*-flanked version of exons 5 and 6 of *Brca1* (*Brca1*$^{co/-}$) and a Cre transgene driven by a T-cell specific promoter from the tyrosine kinase gene *Lck* (Table 1.3 #3) (Hakem, 1996; Mak, 2000). The authors speculated that if *Brca1*-deficient cells had a defect in NHEJ, these mice would have a reduced or absent number of mature T-cells. *Brca1*$^{co/-}$, *Lck*-Cre mice did have a 90% reduction in T-cell numbers, but V(D)J recombination appeared to be unaffected (Mak, 2000). Additionally, a second group studying the

development of lymphomas in *Brca1*$^{\Delta X.11/\Delta X.11}$*, p53*$^{-/-}$ mice (Table 1.3 #7) have shown that although these mice develop tumours at an early age, mature T and B cells are not depleted, and V(D)J recombination appears to occur normally (Xu, 2001b; Bachelier, 2003).

However, one group has persistently documented a decreased efficiency of NHEJ in *Brca1*$^{-/-}$*, p53*$^{-/-}$ MEFs generated from E 9.5 *p53*$^{-/-}$*, Brca1*$^{\Delta X.11/\Delta X.11}$ embryos (*Brca1* allele from Table 1.3 #4). In a cell-free end-joining assay, extracts from these *Brca1*$^{-/-}$*, p53*$^{-/-}$ MEFs were less efficient at end-rejoining than an extract from *p53*$^{-/-}$ MEFs, and end-joining could be impaired in a wildtype cell extract by addition of antibodies against Brca1 (Zhong, 2002a). The same MEFs have been used in a variety of other NHEJ assays: an I-SceI reporter-cassette assay, an assay to monitor the re-annealing of a linearized plasmid, and a retroviral infection assay (Zhong, 2002b). Retroviral infection of cells defective in NHEJ is a cytotoxic event; NHEJ appears to mediate circularization of non-integrated virus, and an inability to circularize non-integrated copies of virus may result in cell death triggered by the presence of excess DNA free ends (Daniel, 1999; Daniel, 2001; Li, 2001). Regardless of the assay, *Brca1*$^{-/-}$*, p53*$^{-/-}$ MEFs showed a significant decrease in NHEJ activity when compared to *p53*$^{-/-}$ MEFs, although some assays indicated that the defect may be in precise end-joining, not overall end-joining (Zhong, 2002a; Zhong, 2002b). While critics may point out that measuring NHEJ efficiency is not as straightforward as measuring HRR efficiency (Ferguson and Alt, 2001), a variety of assays have been performed. One caveat to these experiments is that there is a possibility of additional mutations in the *Brca1*$^{-/-}$*, p53*$^{-/-}$ MEF line. Given the still-conflicting evidence, it is fair to say that more studies on the role of BRCA1 in NHEJ are still required.

1.11.4 Repair of Mutated Bases

1.11.4.1 An overview of base repair

Mutated bases, such as pyrimidine dimers from UV exposure, oxidative lesions such as 8-oxo-guanine from oxygen free-radical exposure, or replication errors, are repaired by three different pathways – base excision

repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). Although some of the proteins involved in MMR co-immunoprecipitate with BRCA1 (Wang, 2000b), experiments involving BRCA1 to date have focused mainly on BER and NER. These two pathways share some substrates, but differ in their method of action. NER uses a core set of proteins to recognize many lesions, and is generally involved in the repair of bulky lesions such as UV-induced photoproducts. In contrast, BER uses lesion-specific proteins to recognize damage, and tends to repair oxidative lesions. NER is further subdivided into transcription coupled repair (TCR), which preferentially and rapidly repairs the transcribed strand of active genes, and global genomic repair (GGR), which repairs the remainder of the genome and is slower than TCR (Figure 1.16). Recent evidence shows that BER also has a TCR component (reviewed in Svejstrup, 2002).

Repair of mutated bases is an important process for maintaining genome stability, and mutations in repair pathways manifest in clinical syndromes such as Xeroderma Pigmentosum (XP) and Cockayne's Syndrome (CS). XP patients have an overall NER deficiency (except for XPC types, which have only a mutation in GGR, and are competent for TCR), and consequently have a very high incidence of skin cancers from failure to repair UV-induced damage. CS patients, on the other hand, have a specific deficiency in the TCR component of NER, but since they can still repair lesions through GGR, they do not have an increased incidence of skin cancer from UV exposure. However, they do have other, severe, symptoms, likely because of a lack of repair of oxidative lesions on the transcribed strand (TCR-BER) (reviewed in de Boer and Hoeijmakers, 2000; Svejstrup, 2002).

1.11.4.2 BRCA1 and Base Repair

Experimental data implicating BRCA1 in the repair of mutated bases is not as abundant as the DSBR data, but there is evidence for its involvement. BRCA1 is upregulated, hyperphosphorylated, and located in damage-induced foci following UV exposure (Scully, 1997b; Clarkin, 2000; Okada and Ouchi, 2003). Upregulation of BRCA1 appears to result in upregulation of the genes

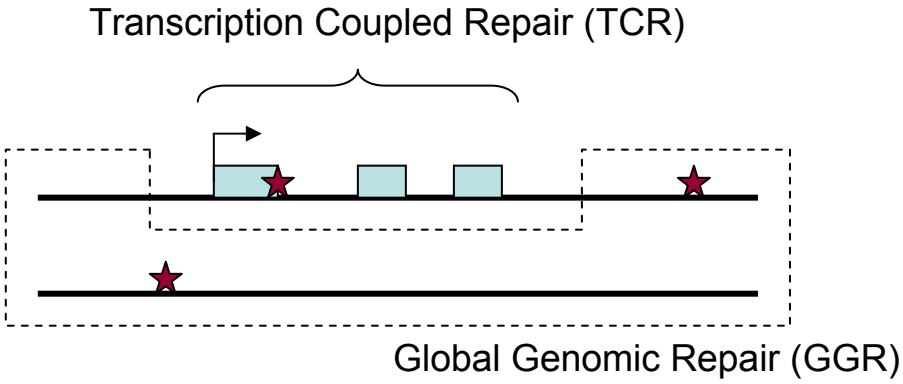


Figure 1.16: Base repair on transcribed or non-transcribed strands of DNA. Schematic showing that mutations (red stars) in actively transcribed genes are repaired by transcription-coupled repair (TCR), while those elsewhere are repaired by the global genomic repair (GGR) pathway.

p21 and *GADD45*, as described in section 1.5.3.1 (Somasundaram, 1997; Amundson, 1998; Harkin, 1999; MacLachlan, 2000b).

The role of BRCA1 in NER has been investigated using tetracycline-controlled overexpression of *BRCA1* in $p53^{+/+}$ or $p53^{-/-}$ human cell lines (Harkin, 1999). In $p53^{-/-}$ cells, UV-induced lesions are repaired efficiently by TCR, but cannot be repaired by GGR. However, when *BRCA1* expression was induced in $p53^{-/-}$ cells, UV-induced lesions on the non-transcribed strand were repaired efficiently. Induction of *BRCA1* in $p53^{+/+}$ cells did not significantly change the amount of repair on either strand. Induction of BRCA1 expression in $p53^{-/-}$ cells led to normal upregulation of the *GADD45* gene following UV exposure. While these results are interesting, this study was based on overexpressing *BRCA1*, and the role of endogenous levels of BRCA1 in this process should still be investigated, especially in light of a recent study which shows that while *BRCA1* is indeed upregulated shortly after UV exposure, expression levels drop again about an hour after UV exposure. Constant expression of *BRCA1* after damage may not accurately model the consequences of this downregulation (Harkin, 1999; Okada and Ouchi, 2003). A second group has shown that mouse $Brca1^{\Delta X.11/\Delta X.11}$, $p53^{-/-}$ MEFs may be hypersensitive to UV exposure when compared to $Brca1^{+/\Delta X.11}$, $p53^{-/-}$ MEFs (allele from Table 1.3 #5); the difference in sensitivity between the two genotypes was statistically significant only at some of the UV doses tested (Cressman, 1999a).

Base mutation can also occur following exposure to oxidative stresses, such as oxygen free-radicals. Common oxidative lesions include thymine glycols and 8-oxo-guanine (Collins, 1999). HCC1937 cells appear to be deficient in TCR of an 8-oxo-guanine lesion transfected into the cells on a plasmid, but are competent to repair the same lesion on the non-transcribed strand (Le Page, 2000b). 8-oxo-guanine lesions are generally repaired by BER, but repair of the mutation in actively transcribed genes occurs through a TCR pathway (Le Page, 2000a). $Brca1^{\Delta X.11/\Delta X.11}$ ES cells (Table 1.3 #5) appear to be hypersensitive to oxidative damage from H_2O_2 exposure, and some evidence indicates that this may result from a deficiency in TCR (Gowen, 1998; Gowen, 2003). $Brca1^{\Delta X.11/\Delta X.11}$, $p53^{-/-}$ MEFs are also hypersensitive to

H_2O_2 exposure when compared to *Brca1*$^{+/\Delta X.11}$, *p53*$^{-/-}$ MEFs (allele from Table 1.3 #5) (Cressman, 1999a).

Evidence from these studies indicates that BRCA1 plays a role in the repair of base damage, but more work is necessary before this role can be precisely defined. In particular, it will be important to investigate the role of BRCA1 in repair when *p53* is not also mutated. Recent evidence suggests that p53 may regulate the level of *BRCA1* expression following DNA damage. The absence of both p53 and BRCA1 may cause synergistic effects which would confound the conclusions of some of these studies (Somasundaram, 1999; MacLachlan, 2000a).

1.11.5 In summary

BRCA1 clearly plays a role in DNA repair. However, the results of the variety of assays (using a myriad of cell lines and BRCA1 mutations) used to reach this conclusion are not without contradiction, and more work is needed to clearly define what roles BRCA1 plays in the repair of base lesions and DNA DSBs.

1.12 THE AIMS OF THIS PROJECT

1.12.1 Existing murine alleles of *Brca1*

A number of mouse *Brca1* knockout alleles and mouse models had been generated when this study began, most designed to investigate the consequences of the loss of *Brca1* on the tumourigenic process. The main findings were that homozygous mutants were embryonic lethal while heterozygotes were normal and had no increased predisposition to tumours (Hakem, 1996; Liu, 1996; Ludwig, 1997; Shen, 1998; Hohenstein, 2001). Embryonic lethality in homozygous mutant ES cells or early embryos compared to tumourigenesis in mature breast and ovarian tissues in human carriers of *BRCA1* mutations can best be described as paradoxical: it seems contradictory but is compatible with the definition of *BRCA1* as a caretaker

tumour-suppressor gene. In mature cells of *BRCA1* mutation carriers, the second *BRCA1* mutation is acquired somatically, probably through LOH. This results in genomic instability leading to mutations in other genes which in turn leads to tumourigenesis. In homozygous *Brca1* mutant embryos, the second *Brca1* hit has already occurred, and the resulting genomic instability is likely incompatible with the massive amounts of growth and differentiation needed to generate a viable mouse. SKY analysis done on embryos homozygous for a *Brca1* Δ*X.11* allele supports this idea by showing that these embryos do indeed have an increased number of chromosomal defects. The addition of a *p53* mutation, which increases the time before $Brca1^{-/-}$ embryos undergo growth arrest, exacerbates the extent of genomic rearrangement. This indicates that the *p53* mutation does not mitigate the *Brca1*-deficient phenotype, but more likely allows damaged cells to bypass a cell-cycle checkpoint for genomic integrity (Shen, 1998).

1.12.2 A conditional *Brca1* ES cell system

These previously-generated mouse models were useful in helping to define the role of *BRCA1* in genomic stability. However, even the conditional mutations of *Brca1*, which were just emerging as this project began, had limited use in revealing the genetic or biochemical pathways behind the caretaker role of *Brca1*. Instead of looking solely at the mouse model, it seemed practical to try and address functional questions in ES cell lines, where additional genetic manipulations could be carried out using familiar and well-tested techniques. As in embryos, *Brca1* appears to be necessary for ES cell viability.

In order to study *Brca1* in ES cells, a conditional *Brca1* system was generated, consisting of one knockout allele and one conditional allele ($Brca1^{co/-}$) (Figure 1.17). These 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 (Table 1.3 #1) (Ludwig, 1997). Targeting exon 2 represented a departure from the numerous groups who produced and were studying exon 11 knockouts. Since *Brca1* Δ*X.11* is a

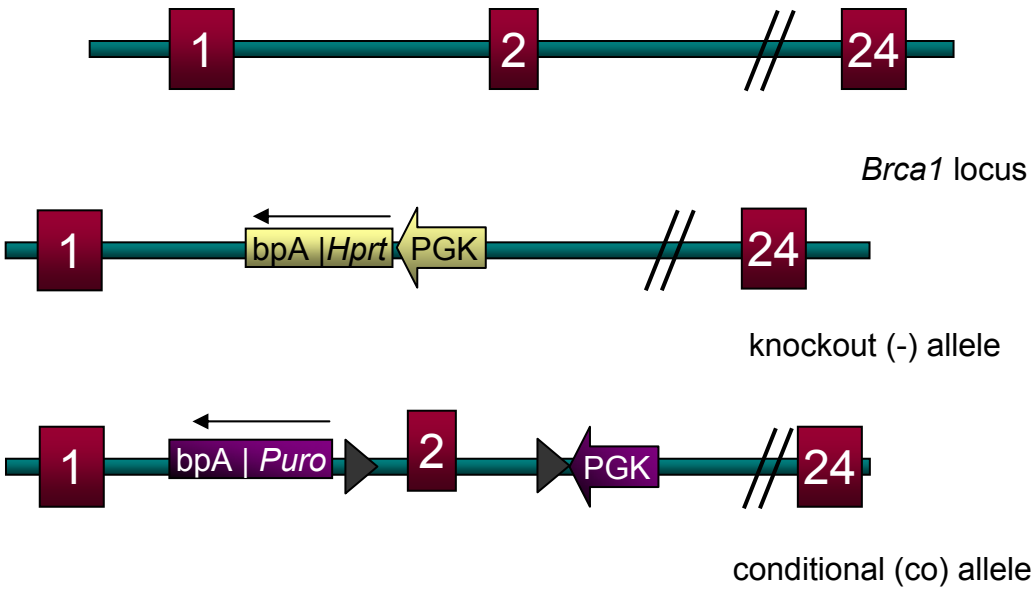


Figure 1.17: Overview of the *Brca1* conditional ES cell alleles. Knockout (-) and conditional knockout (co) alleles of *Brca1*, both targeting exon 2. The knockout allele carries a *Hypoxanthine phosphoribosyltransferase* (*Hprt)* mini-gene which confers HAT resistance, and the conditional allele carries a bipartite *Puromycin* (*Puro)* gene designed to allow puromycin-mediated selection of the allele following Cre-mediated excision. Grey triangles represent *loxP* sites.

natural splice isoform, predicted to share at least some function with full-length *Brca1*, its presence in the ES cells might confuse the findings of a functional screen.

1.12.2.1 A gene trap suppressor screen

Brca1$^{co/-}$ ES cells were primarily generated for use in a genome-wide gene trap screen for suppressors of *Brca1*. There is some support for the notion that suppressors of *Brca1* exist: one mouse study demonstrated that mice homozygous for a truncated Brca1 protein were born at the expected Mendelian ratios only on certain strain backgrounds (Ludwig, 2001). Although a report exists describing a woman who is homozygous for a cancer-related *BRCA1* mutation (Boyd, 1995), this finding has been disputed and attributed to a PCR error (Kuschel, 2001).

The suppressor screen consists of two steps; genome-wide mutagenesis and subsequent selection for viable cells carrying functional suppressors. Before recombinase-mediated deletion of the second copy of *Brca1*, a genome-wide mutagen in the form of a retrovirally-delivered gene trap is introduced into conditional ES cells. This gene trap carries a splice acceptor upstream of an antibiotic resistance gene (*β-geo*, a fusion of the *Neo* gene, which encodes resistance to the drug G418, and *β-galactosidase*) which lacks a translational start site. Integration of the gene trap into the intron of a gene is expected to result in splicing of *β-geo* into the transcript, mutating the gene by truncation, tagging the truncated gene with the inserted trap, and allowing selection and/or screening of trapped cell lines (Figure 1.18).

Following gene trapping, the conditional allele undergoes Cre-mediated deletion. Deletion of the conditional allele is a selectable event, as the conditional allele was designed with a split *puromycin N-acetyltransferase* (*Puro*) selection cassette around exon 2 (Figure 1.17). ES cells which lack both copies of *Brca1* are expected to be non-viable, but if the gene trap cassette traps a suppressor of *Brca1*, cells carrying that trap should be viable. The screening criteria are highly stringent, for antibiotic resistance markers

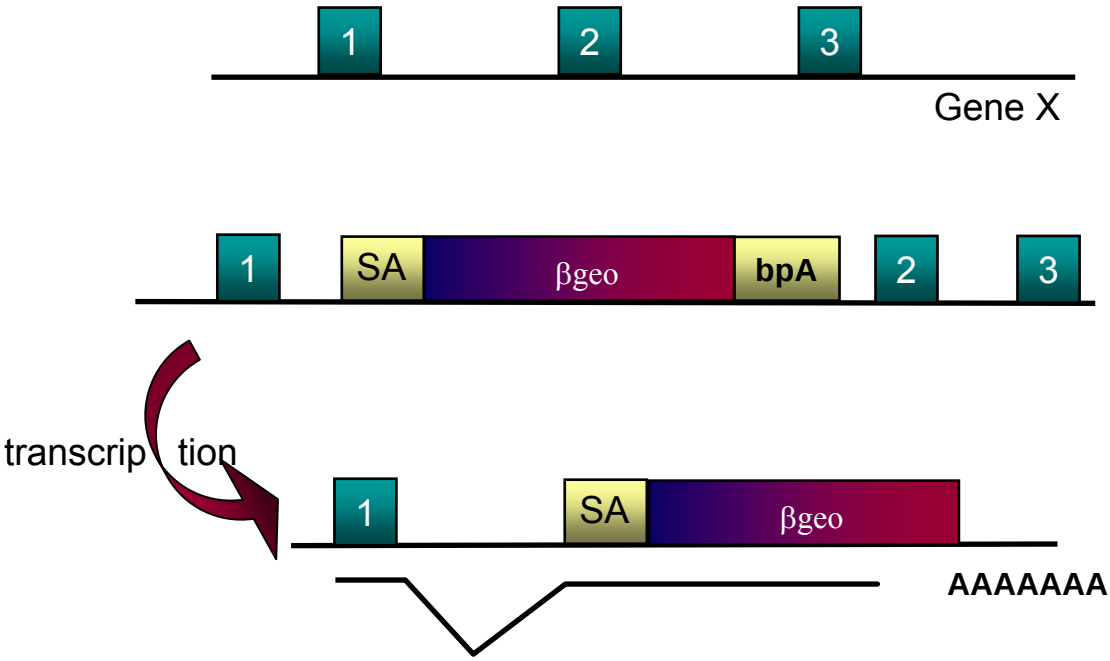


Figure 1.18: Gene trap mutagenesis. A splice-acceptor (SA) *β-geo* (a fusion between the *Neomycin phosphotransferase* (*neo*) gene which codes for resistance to G418 and *β-galactosidase*) gene trap is randomly integrated into the genome. Inclusion of the gene trap cassette in an intron results in splicing of *β-geo* into the transcript, both tagging (with *Neo*) and mutating (through truncation) the trapped gene.

are carried by both alleles of *Brca1* and by the gene trap, and only functional suppressors should result in viable cells (Figure 1.19).

1.12.2.2 Trapping recessive suppressors

One drawback to this screen as described is that it would only be expected to trap dominant suppressors. Therefore, a modification was made to allow screening for recessive genes. A colleague in the lab, Guangbin Luo, has generated a mouse knockout model of Bloom's Syndrome (*Blm*$^{-/-}$). Bloom's Syndrome is a rare recessive syndrome which results from mutation of the RecQ helicase homologue BLM. Mutation of the BLM helicase results in an increased frequency of mitotic recombination and LOH, both in human patients and in a mouse *Blm*$^{-/-}$ model. Guangbin determined that the mitotic recombinational rate in *Blm*$^{-/-}$ ES cells was approximately 20-fold higher than that of wildtype cells (Luo, 2000). This increase in the rate of mitotic recombination can be exploited for screening for recessive mutations: given sufficient doubling times in culture, cells carrying one copy of a gene trap should undergo LOH at that locus. Half of such events should result in homozygosity of the gene trap at the given locus. By these means, recessive suppressors can be trapped in essentially the same screen as the one for dominant suppressors.

The main condition for either suppressor screen was that both knockout alleles of *Brca1* had to behave as null alleles (once fully deleted), in order to allow for selection by cell viability.

1.12.3 Tumourigenesis studies

Besides being used for the suppressor screen, both knockout alleles of *Brca1* were used to generate mice for tumourigenesis studies. The standard knockout was used in conjunction with a Bloom's Syndrome knockout background. It was thought that using this background might accelerate loss of the wildtype copy of the *Brca1* gene and subsequently accelerate tumourigenesis. The conditional allele was used in a tumourigenesis study in

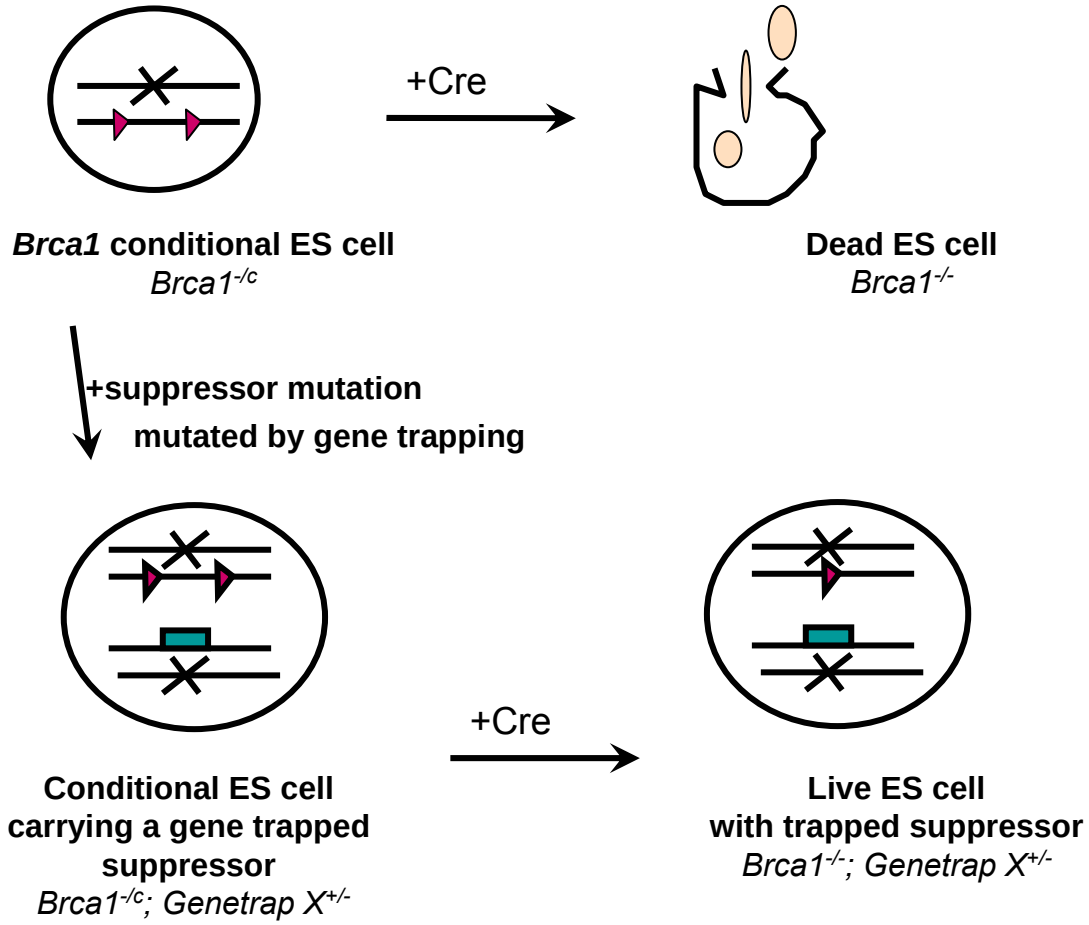


Figure 1.19: General overview of the gene trap suppressor screen in conditional *Brca1* ES cells. Complete loss of *Brca1* in ES cells is expected to be a lethal event. A conditional ES cell system, with one knockout and one *loxP*-flanked (red triangles) conditional allele, is subjected to genome-wide mutagenesis though gene trapping, then the conditional allele is excised by Cre. Only cells carrying a suppressor mutation will be viable following this loss of *Brca1*.

conjunction with Cre transgenes driven either by a breast-specific or ubiquitously-expressed promoter.

1.12.4 An overview of the chapters in this work

1.12.4.1 Generation of knockout alleles

Chapter 3 discusses the first goal of this project, which was to generate the two *Brca1* knockout alleles and target them into ES cells. Unexpectedly, the conditional allele generated in this study did not behave as a null allele following Cre-mediated deletion. This precluded the use of the conditional ES cells in a suppressor screen, but provided a new tool for studying *Brca1* function, as ES cells carrying two copies of this recombined conditional allele were viable. As this allele was predicted to give rise to a protein which lacks the N-terminal RING domain, it was named *gollum* (*gol*). Chapter 4 describes the generation of mice from ES cells carrying knockout alleles of *Brca1* and the results of the tumourigenesis studies performed using these mice.

1.12.4.2 DNA damage and the gol allele

The second aim of this work, discussed in Chapter 5, was to determine the response of *gol/gol* and *+/gol* ES cells to various forms of DNA damage. A large body of experiments provides evidence that *Brca1* is involved in DNA repair, and it was expected that having a mutant allele which specifically lacked one part of the protein would be useful in determining the role that domain had in the response to DNA damage. Immunolocalization was also performed and it was determined that the protein produced from the *gol* allele is able to localize to the nucleus and forms both S phase and DNA damage-induced nuclear foci. The localization of the mutant protein was particularly important in light of the discovery that Bard1, which interacts with Brca1 at the RING domain, is a nuclear chaperone and retention protein for Brca1, as a RING-less version of Brca1 might be not be expected to localize to the nucleus.

1.12.4.3 Molecular characteristics of the gol allele

The viability of the *gol* allele was unexpected, especially in light of the fact that the standard knockout generated in this study deletes the same exon. The third goal of this study, described in Chapter 6, was to molecularly characterize the *gol* allele, including determining the nature of the protein produced from the *gol* allele and investigating potential changes in *Brca1* RNA or protein levels in cells carrying this allele. It was also of interest to determine if Bard1 was able to bind to Brca1, in light of the findings that Brca1 localized to the nucleus in *gol/gol* cells. The findings suggest that the *gol* allele may be a useful tool for many further experiments.

CHAPTER TWO:

MATERIALS AND METHODS

2.1 VECTORS

2.1.1 Targeting vectors:

2.1.1.1 Brca1-Hprt-TV (targeting vector)

The *Brca1*-Hprt-TV was designed to replace exon 2 with a *hypoxanthine phosphoribosyltransferase* (*Hprt*) minigene transcribed in the opposite direction from transcription of the *Brca1* allele (Figure 3.1a). A previously-published *Brca1* targeting vector (*Brca1*-Neo-TV), which replaces exon 2 with a *Neomycin phosphotransferase* (*Neo*) gene driven by the *phosphoglycerate kinase* gene promoter (PGK), was a gift from Drs. Thomas Ludwig and Argiris Efstratiadis (Ludwig, 1997). The *Neo* cassette from this vector was excised using a *Sal*I/*Xho*I$_{partial}$ digest, and the remaining ~14.4 kb *Sal*I/*Xho*I fragment was re-ligated. A 2.6 kb PGK-*Hprt*-bpA minigene, a gift from Anton Berns (van der Lugt, 1991), was cloned into a *Cla*I site left behind at the PGK-*Neo*-bpA insertion site. The *Cla*I-flanked *Hprt* minigene was generated by sequential cloning of the minigene into the cloning vectors pBS+ (Stratagene) and pSP73 (Promega). The finished vector consists of a 2.1 kb 5' arm and a 7.8 kb 3' arm flanking the PGK-*Hprt*-bpA which replaces a ~700 bp fragment containing exon 2. A herpes simplex virus type I *thymidine kinase* (HSV-*tk*) gene is included at the 3' end as a negative selection marker against random integration (Wigler, 1977; Borrelli, 1988)

2.1.1.2 Brca1-cond1-TV

The *Brca1*-cond1-TV was designed to flank exon 2 with *loxP* sites and has a *loxP*-flanked *Neo* selection cassette (Figure 3.4). It was constructed from three component vectors:

1. Backbone/arms: ~10 kb of *Brca1* genomic sequence (from 129 /Sv genomic DNA) containing exons 1 and 2 and most of intron 2 was cloned *Sal*I/*Eag*I into *Not*I/*Sal*I-digested pSK+ vector (Stratagene). This construct was partially digested with *Bam*HI to yield a linear, 13 kb product.
2. *Puromycin* (*Puro*)-containing cassette: an *Xho*I/*Pst*I fragment containing the *Puro* coding sequence and a poly-A signal was cloned

into a pSK+ vector containing an oligonucleotide linker containing *Pst*I and *Sal*I sites. A 100 bp *Pst*I-fragment containing a *loxP* site, excised from p*loxP*-PGK-*Neo*-bpA-*loxP* (a *Neo* gene flanked by *loxP* sites, a gift of Dr. Richard Behringer from the MD Anderson Cancer Center in Houston, TX) was added immediately downstream of the *Puro* coding region. The finished cassette (~1 kb) was excised from the vector using *Bgl*II and cloned into the *Bam*HI-digested backbone vector from #1, immediately upstream of exon 2. The result of this ligation was re-linearized with *Bam*HI.

3. *Neomycin*/PGK-containing cassette: A 1.7 kb *Not*I/*Xho*I fragment from p*loxP*-PGK-*Neo*-bpA-*loxP* was cloned into cloned into a pSK+ vector containing an oligonucleotide linker containing *Not*I and *Sal*I sites. A *Sal*I/*Xho*I fragment containing the PGK promoter (excised from pPGK-*Puro*-bpA) was cloned into an *Xho*I site 3' of the *Neo* cassette. The finished cassette (2.2 kb) was excised from the vector using a *Bam*HI/*Bgl*II$_{partial}$ digest, and cloned into the *Bam*HI-linearized vector from #2. The insertion was 2.1 kb downstream of exon 2 .

A 2.1 kb *Xho*I/*Sal*I-flanked HSV-*tk* gene was cloned into a unique *Sal*I site at the 3' end of the completed vector. The *loxP* sites of the vector were checked using a Cre enzyme (a gift of Dr. Steven Elledge, Baylor College of Medicine, Houston, TX).

2.1.1.3 Brca1-fixPuro-TV

The *Brca1*-fixPuro-TV (Figure 3.6) was designed to replace the *Puro* coding region of the *c1* allele with a corrected version. The 2.1 kb 5' arm of *Brca1*-cond1-TV was excised with *Hind*III and *Not*I and three-way cloned with a *Hind*III/*Xba*I-excised *loxP*-PGK-*Neo*-bpA-*loxP* cassette and a *Nhe*I/*Not*I fragment containing 2.3 kb of 3' *Brca1* sequence, the HSV-*tk* gene, and the vector backbone (this fragment was taken from a cloning intermediate of *Brca1*-cond1). The vector was checked by sequencing.

2.1.1.4 Brca1-addPGK-TV

The *Brca1*-addPGK-TV was designed to add a PGK promoter to the *c1* allele (Figure 3.7) and was the product of a three-way ligation of:

1. 5.4 kb *Brca1* genomic DNA was amplified from mouse AB2.2 ES cell genomic DNA using the Long Expand PCR System (Roche) and with primers carrying *Xho*I or *Not*I sites. Forward primer: 5' gat aca gcg gcc gcg tgt gga tgc tgg gaa ttg aac cttt g 3'; reverse primer: 5' gct act ctc gag gag aca ggc tag aca cca aag gaa g 3'. Cycling conditions were: 92°C for 2 min; 10 cycles of 92°C for 10 sec, 62°C for 30 sec, 68°C for 5 min; 15 cycles of 92°C for 10 sec, 62°C for 30 sec, 68°C for 5 min (+20 sec extra per cycle); 68°C for 7 min. The amplified product was digested with *Xho*I and *Not*I and cloned into a *Xho*I/*Not*I-digested pSK+ vector. The resulting vector was cut with *Bam*HI.
2. The PGK promoter was amplified from the pPGK-*Neo*-bpA plasmid by PCR using the Roche Expand High Fidelity PCR System and primers carrying *Bgl*II or *Eco*RI sites. Forward primer: 5' gca tcg aag ctt aga tct ggg gag gcg ctt ttc cca ag 3'; reverse primer: 5' gct gca gaa ttc gca ggt cga aag gcc cgg ag 3'. Cycling conditions were: 94°C 2 min; 10 cycles of 94°C for 15 sec, 62°C for 30 sec, 72°C for 45 sec; 15 cycles of 94°C for 15 sec, 62°C for 30 sec, 72°C for 45 sec (+5 sec extra per cycle); 72°C for 7 min. The amplified product was digested with *Bgl*II and *Eco*RI.
3. A 1.8 kb *Bam*HI/*Eco*RI–flanked *loxP*-PGK-*Neo*-bpA-*loxP* cassette.

The final vector was checked by sequencing and the *loxP* sites were checked using a commercial preparation of Cre enzyme (Clontech).

2.1.1.5 Brca1-cond2-TV

The *Brca1*-cond2-TV vector was constructed for archive purposes and as a step in the cloning of *Brca1-gollum* (Figure 3.3b). It flanks exon 2 with *loxP* sites and a split *Puro* cassette, and has a *loxP*-flanked *Neo* cassette. The *Brca1*-fixPuro-TV was transformed into the Cre-recombinase-expressing

BNN132 *E. coli* cells (a gift of Dr. Steve Elledge at Baylor College of Medicine, Houston, TX) to remove the *loxP*-flanked *Neo* selection cassette. An 8.6 kb *Sal*I/*Bam*HI$_{partial}$ fragment from this Cre-excised plasmid (containing the vector backbone, 2.1 kb 5' arm, *Puro*-bpA cassette, and a 2.8 kb region containing exon 2) was ligated to a 5 kb *Bam*HI/*Xho*I$_{partial}$ – digested section of *Brca1*-addPGK-TV (containing the *Neo* selection cassette, the reversed PGK promoter intended to drive the *Puro* gene, and the 2.8 kb 3' arm). The final vector was checked by sequencing.

2.1.1.6 Brca1-gollum-TV

Brca1-gollum-TV replaces exon 2 of *Brca1* with a *Puro* cassette (Figure 3.12). *Brca1*-cond2-TV DNA was transformed into BNN132 cells. Mini-preparation of resulting colonies from this transformation were screened for plasmids with no remaining *loxP*-flanked regions.

2.1.1.7 Gdf-9 targeting vector (Gdf9-TV)

The replacement targeting vector for the *Growth and Differentiation Factor-9* (*Gdf-9*) gene was a gift from Marty Matzuk (Baylor College of Medicine, Houston, TX (Dong, 1996)). It carries an *Hprt* selection cassette (Figure 5.6).

2.1.1.8 Melk targeting vector (Melk-TV)

A *maternal embryonic leucine zipper kinase* (*Melk*) insertion vector was isolated from a targeting vector library by a colleague, Jyh-Yih Chen. It carries a *Neo* selection cassette (Figure 5.6).

2.1.1.9 PGK-test vectors

Two *Puro* vectors were constructed from a standard pPGK-*Puro*-bpA vector (which also served as the positive control): *Puro*-bpA (no promoter) was generated using a *Sal*I/*Xho*I$_{partial}$ –digested pPGK-*Puro*-bpA vector; a 4.0 kb fragment was isolated and re-ligated. Reversed-PGK-*Puro*-bpA (revPGK-*Puro*-bpA) was generated by excising the PGK promoter from pPGK-*Puro*-bpA by *Bgl*II/*Sal*I digestion, and cloning in a *Bam*HI/*Sal*I-flanked reversed PGK promoter from another pPGK-*Puro*-bpA plasmid.

2.1.1.10 Vectors for transient expression of Cre in ES cells

A CMV-Cre plasmid (pOG231-Cre, a gift from Steve O'Gorman) was prepared by standard alkaline lysis and cesium chloride gradient purification. 25 μg of uncut CMV-Cre was used for transient transfection. The Turbo-Cre (Genbank Accession Number AF334827) plasmid was a gift of the Ley lab at the Washington University School of Medicine. 20 μg of uncut Turbo-Cre was used for transient transfection.

2.1.1.11 Brca1 and Bard1 fusion-protein expression vectors for transient transfection

Four Brca1 N-terminal GST (glutathione-S-transferase) fusion protein vectors were constructed. Partial *Brca1* sequences were amplified by PCR from wildtype AB2.2 ES cell cDNA using forward primers in Brca1 exons 2, 3, 5, or 6 and an exon 10 reverse primer. The exon 5 primer begins after the exon 5 ATG sequence, the exon 6 one after the first exon 6 ATG sequence (Figure 6.12). Amplification was performed using the Expand High-Fidelity PCR kit (Roche). Cycling conditions were: 94°C for 5 min; 10 cycles of: 94°C for 15 sec, 60°C for 30 sec, 72°C for 1 min; 15 cycles of: 94°C for 15 sec, 60°C for 30 sec, 72°C for 1 min (+5 sec extra per cycle); 72°C for 10 min. Primers: Exon 2 forward (670 bp product): 5' atg cta gga tcc atg gat tta tct gcc gtc caa att caa g 3'; Exon 3 forward (560 bp product): 5' atg cta gga tcc ttt gga act gat caa aga acc tgt ttc 3'; Exon 5 forward (520 bp product): 5' atg cta gga tcc atg ctg aaa ctt ctt aac cag aag aaa gg 3'; Exon 6 forward (400 bp product): 5' atg cta gga tcc atg gct gct ttt gag ctt gac acg gg; and Exon 10 reverse: 5' atg cta gga tcc tta ctc ttc tgc aga gtg cag ctt gc 3'. All primers carry a *Bam*HI site. Amplified products were digested with *Bam*HI and cloned into the *Bam*HI site of the GST fusion vector pEBG (derived from pEF-BOS (Sanchez, 1994)); the fusion protein is expressed under the control of the human *polypeptide chain elongation factor 1α* (*Ef-1α*) gene promoter.

An N-terminal c-myc–tagged Bard1 fusion protein vector was generated by PCR amplification of a 2.3 kb, full-length *Bard1* transcript from wildtype ES cell cDNA using the Expand High-Fidelity PCR kit (Roche). Cycling

conditions were: 94°C for 5 min; 10 cycles of: 94°C for 15 sec, 58°C for 30 sec, 72°C for 2 min; 15 cycles of: 94°C for 15 sec, 58°C for 30 sec, 72°C for 2 min (+5 sec extra per cycle); 72°C for 10 min. Forward primer: 5' gat cga ctc gag acc acg ccg gcc gcc gag ggt c 3'; reverse primer: 5' cta gct ctc gag tca gct gtc aag agg aag caa ttc 3'. These primers both carry an *Xho*I site. The amplified product was cleaved with *Xho*I and cloned into the *Sal*I site of a pCMV-myc expression vector (Clontech), which expresses the fusion protein under the control of a CMV promoter. Final preparation of all fusion protein vectors was done using a Qiagen mini-prep kit (Qiagen Ltd.).

2.2 CELL CULTURE

2.2.1 Cell culture conditions:

ES cell culture was performed basically as described (Ramirez-Solis, 1993). Briefly, AB2.2 (129 S7/SvEv Brd-*Hprt*$^{b-m2}$) wildtype ES cells were cultured on SNL6/7 fibroblast feeder layers, mitotically arrested by γ-irradiation, in M-15 (knockout Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/Invitrogen) supplemented with 15% foetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin, 40 μg/ml streptomycin, and 100 μM β-mercaptoethanol (β-ME)). Medium was changed daily. Cells were cultured at 37°C with 5% CO_2. Cells were subcultured every 2-3 days at ratios of 1:2 to 1:10.

Differentiated ES cells and embryoid bodies were cultured in M-10 (knockout Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/Invitrogen), supplemented with 10% FBS, 2 mM glutamine, 50 U/ml penicillin, and 40 μg/ml streptomycin). Cells were cultured at 37°C with 5% CO_2. The medium was changed every 2-3 days. Cells were subcultured at ratios of 1:2 to 1:5.

Feeder-free medium is M-15 as described above supplemented with leukocyte inhibitory factor (LIF). LIF amount was titrated by batch; LIF was a gift of Patrick Biggs of the Wellcome Trust Sanger Institute. This medium was used to grow ES cells undergoing FISH or cell cycle analyses.

The Phoenix ecotropic retroviral packaging cell line (derived from the human embryonic kidney 293T line), generated by Gary Nolan of Stanford University, California, USA (Grignani, 1998), was obtained from the American Tissue Culture Collection (Manassas, Virginia, USA). Cells were cultured essentially as described, in M-10 at 37°C with 5% CO_2 (Nolan).

2.2.2 Generation of embryoid bodies

Generation of embryoid bodies from ES cells was performed as described (Robertson, 1987). Briefly, confluent wildtype or *gol/gol* ES cells were passaged onto 90 mm gelatinized plates (tissue culture plates treated with 0.1% (w/v) gelatin for ~2 hours, this provides a substrate for the cells to attach to) at high density to remove feeder cells. After 30 minutes, the supernatant (composed mostly of ES cells in suspension) was moved to a fresh gelatin plate and grown until confluent. Cells were very lightly trypsinized for ~2 minutes, and plates were tapped gently to produce “flakes” of cells. The trypsin was quenched with fresh M-10 and the flakes were moved onto non-gelatinized 90 mm bacteriological plates to grow in suspension. Four days later, the small balls of cells which had formed were plated onto gelatinized plates and allowed to attach undisturbed for two days. Following attachment, outgrowth of differentiated cells was rapid. Differentiated cells were collected after 3-5 days and plated into flasks prior to being used for immunolocalization.

2.2.3 Gene targeting and electroporation of targeting vectors

Gene targeting was performed essentially as described (Ramirez-Solis, 1993). In general, 10^7 ES cells were electroporated in a 0.4 cm gap cuvette with 25 µg linearized targeting vector (or uncut Cre plasmid) at 230 volts and 500 µFarads and plated onto a 90 mm feeder plate. Ten days after electroporation, 96 colonies were picked into a 96-well feeder plate and grown until confluent. Plates were split 1:2 onto one feeder plate (frozen at confluence) and one gelatin plate (lysed for genomic DNA extraction at

confluence). Targeted clones were identified by mini-Southern analysis, performed as described (Ramirez-Solis, 1993).

Targeting vector DNA was prepared by standard alkaline lysis followed by cesium gradient purification or purification by Qiagen midiprep column. Unless otherwise indicated, 25 µg linearized vector was electroporated. Table 2.1 and the following sections give details of targeting vector linearization sites, cell selection conditions, genomic digests and probes for mini-Southern analysis, and the alleles generated by successful targeting. Unless otherwise indicated, all electroporations were performed using the protocol above.

2.2.3.1 Brca1-Hprt-TV

The *Brca1*-Hprt-TV was electroporated into wildtype AB2.2 cells. Selection of electroporated cells in hypoxanthine/aminopterin/thymine (HAT) (0.1 mM hypoxanthine, 0.4 µM aminopterin, 40 µM thymidine, from Gibco) was started approximately 24 hours after electroporation and continued for 10 days, followed by release in hypoxanthine/thymidine (HT)–containing medium (0.1 mM hypoxanthine 0.4 µM thymidine, from Gibco) for two days. Cells were concurrently selected in 0.2 µM FIAU (1-(2'-deoxy-2'-fluoro-b-D-arabinofuranosyl)-5-iodouracil) against integration of the HSV-*tk* gene.

The allele generated by this vector is designated $Brca1^{Brdm1}$, and is referred to in this work as "$Brca1^{-}$" or simply "–" when in conjunction with another allele (Figure 3.1). Two +/– cell lines (C2 and D2) were used for subsequent analyses.

2.2.3.2 Targeting $Brca1^{+/-}$ ES cells with the Brca1-Neo-TV

20 µg *Not*I-digested *Brca1*-Neo-TV (Ludwig, 1997) was electroporated into $Brca1^{+/-}$ ES cells, to try and generate double-targeted ES cells. Electroporated cells were selected in medium containing 180 µg/ml G418, 0.2 µM FIAU, and HAT for ten days, followed by two days of HT selection.

Table 2.1: Properties of targeting vectors.

Targeting vector/Cre plasmid	Allele generated/ abbreviation	Exon targeted and vector type	Linear-ization enzyme	Electro-porated into (cell line(s))	Selection	Genomic DNA digest	Probe and sizes of fragments identified	Figure	Clones injected
Brca1-Hprt-TV	*Brca1*^{Brdm1}, *Brca1*$^{-}$	Replacement vector, exon 2	*Pvu* I (2x in vector)	AB 2.2 wildtype	HAT/HT and 0.2 μM FIAU	*Hind* III	Probe A: 10.4 kb (WT), 7.2 (*Brca1*$^{-}$). Probe B: 10.4 kb (WT), 3.2 kb (*Brca1*$^{-}$).		C2 and D2 (both injected, both transmitted)
Brca1-Neo-TV (Ludwig, 1997)	*Brca1*^{Neo}	Replacement vector, exon 2	*Not* I	+/-	G418 (180 μg/ml) and 0.2 μM FIAU	*Hind* III	Probe A: as above.		NA
Brca1-cond1-TV	*Brca1*^{Brdc1}, *c1.* Before excision of selection cassette: *c1(+Neo)*	Conditional vector, exon 2	*Sal* I	AB 2.2 wildtype, +/-	G418 (180 μg/ml)	*Hind* III	Probe A: 10.4 kb (WT), 5.6 kb (*c1+Neo*). Probe B: 10.4 kb (WT), 7.2 kb (*c1(+Neo)*).		NA
+CMV-Cre	*Brca1*^{c1}, *c1*	Removes the *Neo* selection cassette of *c1(+Neo)*		*+/c1(+neo), -/c1(+Neo)*	None	*Hind* III	Probe A: 10.4 kb (WT), 3.8 kb (*c1*). Probe B: 10.4 kb (WT), 7.2 kb (*c1*).		D9 and F2 (F2 transmitted)
Brca1-fixPuro-TV	*Brca1*$^{c1(Puro\ corrected)}$, *c1(puro corrected).* Before excision of selection cassette: *c1(Puro corrected+Neo)*	Correct *Puro* coding region of *c1* allele	*Not* I	*+/c1*, *-/c1*	G418 (180 μg/ml)	NA (colonies pooled for Cre electropora tion)	NA		NA
+CMV-Cre	*Brca1*$^{c1(Puro\ corrected)}$, *c1(puro corrected).*	Removes the *Neo* selection cassette of *c1(puro corrected)*		*+/c1(Puro* corrected), *-/c1(Puro* corrected)	None	*Bam* HI	Probe C: 1.9 kb (WT), 1.9 kb (*Brca1*$^{-}$), 2.7 kb (*c1(puro corrected)*), 4.5 kb (*c1(puro corrected+Neo),* 5.2 kb (*c1*).		

Abbreviations: WT: wildtype. Transmitted: allele was successfully transmitted through the germline. Probe A (5' external): 1.4 kb *Eco*RV/*Not*I *Brca1* genomic fragment. Probe B (internal): 340 bp *Bam*HI/*Nhe*I *Brca1* genomic fragment. Probe C (5' external): 444 bp *Brca1* genomic fragment. Probe D (external): 250 bp *Pst*I/*Xba*I *Brca1* genomic fragment. HAT/HT: selection is 0.1 mM hypoxanthine, 0.4 μM aminopterin, 40 μM thymidine for ten days, then 0.1 mM hypoxanthine 0.4 μM thymidine for two days.

Table 2.1 (cont): Properties of targeting vectors.

Targeting vector/Cre plasmid	Allele generated/ abbreviation	Exon targeted and vector type	Linear-ization enzyme	Electro-porated into (cell line(s)	Selection	Genomic DNA digest	Probe and sizes of fragments identified	Figure	Clones injected
Brca1-add PGK-TV	*Brca1*^{Brdc2}, *c2*. Before excision of selection cassette: *c2(+Neo)*	Add a PGK promoter to the *c1* allele	*Sal* I	*+/c1(Puro* corrected), *-/c1(Puro* corrected)	G418 (180 μg/ml)	*Hind* III	Probe A: 10.4 kb (WT), 7.2 kb (*c2(+Neo)*). Probe B: 10.4 kb (WT), 6.0 kb (*c2(+Neo)*).		NA
+Turbo-Cre (Genbank accession Number AF334827)	*Brca1*^{Brdc2}, *c2*.	Removes the *Neo* selection cassette of *c2(+Neo)*		*+/c2(+Neo)*, *-/c2(+Neo)*	None	*Hind* III	Probe A: 10.4 kb (WT), 7.2 kb (*c2*). Probe B: 10.4 kb (WT), 4.2 kb (*c2*).		NA
+Turbo-Cre	*Brca1*^{Brdm2}, *gol*	Removes *loxP*-flanked exon 2 of *c2* allele		*-/c2*	Puro (3 μg/ml)	*Hind* III	Probe A: 10.4 kb (WT), 7.2 kb (*gol*). Probe B: 10.4 kb (WT), 1.5 (*gol)*		NA
Brca1-cond2	*Brca1*^{Brdc2}, *c2* (vector for archive purposes).	Conditional vector, exon 2 (with split *Puro* cassette)	*Not* I	NA	G418 (180 μg/ml)	NA			NA
Brca1-gollum-TV	*Brca1*^{Brdm2}, *gol*	Replacement vector, exon 2	*Not* I	AB 2.2 wildtype	Puro (3 μg/ml)	*Hind* III (probes A, B). *Xba* I (probe D)	Probe A: 10.4 kb (WT), 7.2 kb (*gol*). Probe B: 10.4 kb (WT), 1.5 (*gol)*. Probe D: 7.8 kb (WT), 4.6 kb (*gol*).		H8 (currently being injected)
Gdf9-TV (Dong, 1996)	*Gdf-9*^{m1}	Replacement vector, exon 2	*Pvu* I	AB 2.2 wildtype, *gol/gol*	HAT/HT	*Bam* HI and *Eco* RV	*Gdf-9* probe: 650 bp *Bam*HI/*Sal*I *Gdf-9* genomic fragment (external probe): 9 kb (WT) or 7 kb (*Gdf-9*^{m1}).		NA
Melk-TV	*Melk*^{Brdm1}	Insertion vector	*Nde* I	AB 2.2 wildtype, *gol/gol*	G418 (180 μg/ml)	*Eco* RV	*Melk* probe: 558 bp, 3' external, PCR-generated probe:15 kb (WT) and 6.7 kb (*Melk*^{Brdm1})		NA

2.2.3.3 Brca1-cond1-TV

The *Brca1*-cond1 DNA was electroporated into AB2.2 and *Brca1*$^{+/-}$ ES cells. Cells were selected in 180 μg/ml G418 for ten days. The allele generated using the *Brca1*-cond1-TV is designated *Brca1*^{Brdc1}. For purposes of this work, this allele is referred to as "*c1*," or, when the *Neo* selection cassette is retained, "*c1(+Neo)*" (Figure 3.4). Several *+/c1(+Neo)* and *–/c1(+Neo)* cell lines were identified.

2.2.3.3.1 Cre excision of the Neo selection cassette in the conditional allele c1(+Neo): 25 μg of uncut CMV-Cre plasmid (pOG231-Cre) was transiently transfected by electroporation into *+/c(+Neo)* or *-/c(+Neo)* ES cell lines. Cells were diluted to 10^3/ml after electroporation, and 900 and 1800 cells were plated onto fresh feeder plates. Colonies were picked after ten days in culture without selection. Several *+/c1* and *–/c1* cell lines were identified.

2.2.3.3.2 Testing the bipartite Puro cassette in +/c1 ES cells: +/c1 ES cells were electroporated with Cre recombinase as described above to assess the efficacy of the split *Puro* cassette. Following electroporation, the entire reaction was plated onto puro-resistant feeders and selected for ten days in medium containing 5 μg/ml puro. The experiment was repeated using medium containing 3.5, 3, 2.5, 2, 1.5, or 1 μg/ml puro. Colonies were selected in culture for ten days, and genomic DNA was extracted from pools of colonies grown at a given dose (Figure 3.5).

2.2.3.4 Brca1-fixPuro-TV

The *Brca1*-fixPuro-TV was electroporated into *+/c1* and *–/c1* ES cell lines. Cells were selected in 180 μg/ml G418 for ten days. G418-resistant colonies were pooled, and 10^7 pooled cells were electroporated with Cre to remove the *Neo* selection cassette as described above. Cells were diluted to 10^3/ml after electroporation and 1000 and 2000 cells were plated onto fresh feeder plates. Colonies were picked after ten days in culture without selection.

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).*" One *-/c1(Puro corrected)* and one *+/c1(Puro corrected)* cell line were used for further analysis (Figure 3.6).

2.2.3.4.1 Testing the bipartite Puro cassette in +/c(Puro corrected) ES cells: +/c1(Puro corrected) ES cells were electroporated with Turbo-Cre to test the *Puro* cassette. The entire reaction was plated onto puro-resistant feeders and selected for ten days in culture in medium containing 3 μg/ml puro.

2.2.3.5 Electroporation of the Brca1-addPGK-TV

The *Brca1*-addPGK-TV was electroporated into *+/c1(Puro corrected)* and *–/c1(Puro corrected)* ES cells. Cells were selected in 180 μg/ml G418 for ten days before colonies were picked. The allele resulting from correction of the *c1(Puro corrected)* allele is designated *Brca1*$^{\mathbf{Brdc2}}$. For purposes of this work, this allele is referred to as "*c2*," or, when the *Neo* selection cassette is retained, "*c2(+Neo)*" (Figure 3.7).

Two representative *–/c2(+Neo)* ES cell lines were electroporated with Turbo-Cre plasmid, as described above, to remove the *Neo* selection cassette. Cells were diluted to 10^3/ml after electroporation, and 1000 and 2000 cells were plated. Colonies were picked after ten days in culture without selection. Two *-/c2* clones (C5 and E6) and were expanded, C5 was used for further analysis (Figure 3.7).

2.2.3.5.1 Checking the bipartite Puro cassette in –/c2 ES cells: -/c2 ES cells were electroporated with Turbo-Cre as described above. The entire reaction was plated onto puro resistant feeders and cultured in medium containing 3 μg/ml puro and HAT for ten days, followed by two days under HT selection.

2.2.3.5.2 Analysis of –/c2 ES cells: Twenty-four puro-resistant colonies resulting from Cre electroporation into *–/c2* ES cells were picked and expanded. *Hind*III-digested genomic DNA from these cells was subjected to Southern blot analysis using Probe B (see Table 2.1). Expected band sizes were: 10.4 kb (wildtype), 3.2 kb (*Brca1*$^{-}$), 4.2 kb (*c2*), or 1.5 kb (*c2*, excised). The excised version of *c2* is designated *Brca1*^{Brdm2} or *Brca1*^{gollum}, referred to in this work as "*gol*" (Figure 3.8).

2.2.3.5.3 Generation of –/c2 daughter cell lines: –/c2 ES cells were plated at low density and grown for ten days in culture. Five single, well-isolated colonies were picked and expanded. Each of the five -/*c2* daughter ES cell lines were electroporated with Turbo-Cre as described above. The entire reaction was plated onto puro-resistant feeders and cultured for ten days in medium containing 3 μg/ml puro. Resulting colonies were stained with methylene blue (2% w/v in 70% ethanol) and counted (Table 3.1). The *–/c2* parental cell line was also electroporated with freshly prepared Turbo-Cre. Following electroporation, 10^3, 10^4 and 10^5 cells were plated onto puro-resistant feeder plates and cultured in medium containing 3 μg/ml puro for nine days. Additionally, 10^4 electroporated cells were grown without selection to give the plating efficiency of electroporated cells (Table 3.2).

2.2.3.6 Generation of gol/gol ES cells

Twenty feeder plates containing 2x10^5 *–/gol* ES cells each were selected in 10 μM 6TG for eleven days to screen for *–/gol* ES cells which had undergone spontaneous LOH at the *Brca1* locus; the low cell density was necessary for efficient selection. 96 colonies were picked and screened by mini-Southern analysis of *Hind*III-digested genomic DNA using Probe B. Expected band sizes were: 1.5 kb (*gol*) or 3.2 kb (*Brca1*$^{-}$). Twelve *gol/gol* cell lines were expanded and two (C11 and D9) were used for further analysis (Figure 3.10).

2.2.3.6.1 FISH analysis of gol/gol ES cells: Fluorescence in-situ hybridization (FISH) was performed on both C11 and D9 *gol/gol* ES cell lines, using the BAC RP23-210E12, which contains the entire *Brca1* genomic region

as well as surrounding sequence. The BAC was labeled with biotin (though incorporation of biotin-16-dUTP) by nick-translation.

Cells were cultured in feeder-free medium on gelatin for several passages before analysis, to eliminate contaminating feeders, then 1 x10^7 ES cells were seeded into a T-25 culture flask. The next morning, 0.1 μg/ml colcemid was added to cells, and incubated at 37°C for 40 minutes. Cells were collected by trypsinization, treated with 75 mM KCl for 10 minutes, and fixed in a 3:1 mixture of methanol:glacial acetic acid. After preparation of metaphase spreads on glass slides, 50 ng labeled BAC probe was hybridized to each slide. BAC probes were detected using two-layer FITC-linked detection (avidin FITC DCS followed by FITC-conjugated anti-avidin D); cells were additionally stained with DAPI. BAC labeling and FISH analysis was performed by Ruby Banerjee of the Sanger Institute.

2.2.3.7 Brca1-gollum-TV

The *Brca1*-gollum-TV was electroporated into AB2.2 wildtype cells and cells were selected for ten days in medium containing 3 μg/ml puro (Figure 3.12). One representative *+/gol* ES cell clones (H8) were expanded for further analysis.

2.2.3.8 Electroporation of a Gdf-9 targeting vector (Gdf9-TV) to test targeting efficiencies in gol/gol ES cells

20 μg of *Pvu*I-linearized *Gdf9*-TV was electroporated into AB2.2 wildtype and two *gol/gol* ES cell lines (Dong, 1996) (Figure 5.6). Electroporated cells were selected for ten days in medium containing HAT, then one 96-well plate of colonies was picked from each electroporation. Cells were released from selection in HT-containing medium for two days. Electroporations were done in triplicate; cells used in each repetition were from separate plates of cells.

2.2.3.9 Electroporation of a Melk targeting vector (Melk-TV) to test targeting efficiencies in gol/gol ES cells

20 μg of *Nde*I-linearized *Melk*-TV was electroporated into AB2.2 wildtype and two *gol/gol* ES cell lines. Electroporated cells were selected in medium containing 180 μg/ml G418 for ten days, then one 96-well plate of colonies was picked from each electroporation (Figure 5.6). Electroporations were done in triplicate; cells used in each repetition were from separate plates of cells.

2.2.3.10 Reversed PGK promoter assay

All three vectors used to test the PGK promoter (PGK-*Puro*-bpA, revPGK-*Puro*-bpA, and *Puro*-bpA) in ES cells were linearized at a unique 3' *Not*I site. 10 μg of linearized plasmid was electroporated into wildtype ES cells as above. Puro selection at 3 μg/ml final concentration was initiated 36 hours post-electroporation; cells were cultured in puro-containing medium for ten days. Resulting colonies were stained with methylene blue and counted. Each electroporation was performed twice, on separate occasions.

2.2.4 Electroporation using the gene trap vector pGT for determination of NHEJ efficiency

NHEJ efficiency was tested using the electroporatable, randomly-integrating gene trap vector pGT (a gift of Dr. William Skarnes of the Wellcome Trust Sanger Institute). This vector carries a *β-geo* gene (a fusion of *Neo* and *β-galactosidase* (*β-gal*) genes) preceded by a splice acceptor from the mouse *engrailed-2* gene. *β-geo* lacks an ATG start site, and must be spliced into a gene before it can be expressed (Figure 1.17).

Gene trapping was performed as described, with some modifications (Skarnes, 2000). Briefly 15μg or 10μg of *Hind*III-linearized plasmid was electroporated into 10^7 ES cells as described previously (Ramirez-Solis, 1993). The experiment was performed in triplicate, using one wildtype and two *gol/gol* ES cell lines and all electroporations within a replicate were done

with the same amount of vector. The amount of vector did not influence the number of colonies formed. Replicates were done on separate days). Cells were cultured in medium containing 180 μg/ml G418 for twelve days before colonies were fixed in 0.5% glutaraldehyde in PBS, and stained overnight in the dark with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). X-gal staining produces blue (*β-gal* expressing) or white (non-expressing) colonies. Total and blue-staining colonies were scored. The average total colony number and standard deviation were plotted using Microsoft Excel. A two-tailed t-test was performed to determine if the difference between results was statistically significant.

2.2.5 ES cell growth curves

Wildtype and *gol/gol* ES cells were plated into 30 mm (6 well) feeder plates at $1x10^5$ cells per well. Every twelve hours for seven days, two wells per cell line were collected by trypsinization and the total cell number was determined using a Coulter counter (Beckman-Coulter). Duplicate wells containing only feeder cells were counted at the first and last time points; these counts were averaged to give the background feeder cell count. This background was subtracted from the average cell number from each time point. Doubling times were calculated from the portion of the growth curve denoting log-phase growth and for the overall time in culture, using the formula $DT=(t_1-t_0)/3.3\ \log_{10}(N_1/N_0)$, where t_0 and N_0 represent the time and number of cells at the first time point, and t_1 and N_1 represent the time and number of cells at the last time point (Figure 5.1).

2.2.5.1 Sorting of ES cells

Wildtype and *gol/gol* ES cells were grown for several passages in feeder-free medium. Cells were seeded into 30 mm plates. Cells in replicate plates were counted at 12-hour intervals to ensure that cells being collected were in log-phase growth. Samples of each cell line were collected by trypsinization at two time points 18 hours apart, washed twice in PBS, and fixed in cold 70% ethanol. Fixed cells were washed twice in PBS and treated with RNase A to

prevent staining of RNA (50 μl of 100 μg/ml). 10 μg propidium iodide was added per sample. Cells were sorted on a flow cytometer; propidium iodide fluorescence was collected above 580 nm after excitation by a 488 nm laser. 20,000 cells were counted. Counts were done in triplicate. Flow cytometry was done by Bee Ling Ng of the Sanger Institute. Analysis of cell-cycle profiles was done using WinMDI 2.8 (written by Joseph Trotter).

2.2.6 Transient transfection of Brca1 or Bard1 fusion protein expression vectors

FuGENE transfection reagent (Roche) was used to transfect Phoenix (293T) cells with Brca1 or Bard1 fusion protein expression vectors. Cells were at ~80% confluence on the day of transfection. FuGENE was mixed with DNA at a 3:1 (v/w) ratio. For pilot assays to check for production of fusion protein (Figure 6.12), 1 x 10^6 cells per well were plated into a 30 mm tissue culture plate one day prior to transfection. 3 μg DNA was used per transfection; 3 μg pPGK-*β-geo*-bpA plasmid was transfected as a positive control to check the transfection efficiency. For co-transfection experiments (Figure 6.13), 3.2 x 10^6 Phoenix cells were plated into 90 mm tissue culture plates one day prior to transfection. 10 μg of each plasmid was transfected; co-transfected cells were given 20 μg of DNA. A non-transfected control was performed in all cases. Transfected cells were fed daily. Protein was harvested 72 hours post-transfection. Staining of Phoenix cells with X-gal for detection of *β-geo* was performed as described in section 2.2.4.

2.2.7 Damage Assays of ES cells

2.2.7.1 γ-irradiation

ES cells were collected by trypsinization and centrifugation, and irradiated in M-15 at 100, 250, 500, 750, or 1000 rads in a MDS Nordion Gammacell 1000 Elite γ-irradiator with a ^{137}Cesium source with a dose rate of 789 rads/minute. Immediately following irradiation, cells were plated into 30 mm feeder plates at three different dilutions (see Table 2.2). Controls were treated identically but not irradiated. Each assay was performed in triplicate. Colonies were

Table 2.2: Numbers of ES cells plated following mutagenesis. Following the indicated types of treatment, ES cells of the indicated genotypes were plated into 6-well (30 mm) plates at the following densities. H_2O_2=hydrogen peroxide. See Section 2.2.7 for further details.

Genotype / Mutagen/dose	Wildtype, +/–, or *–/c2* Cells plated	*–/gol* or *gol/gol* Cells plated
γ-irradiation (rads)		
0	10^2, 10^3, 10^4	10^2, 10^3, 10^4
100	10^2, 10^3, 10^4	10^2, 10^3, 10^4
250	10^3, 10^4, 10^5	10^3, 10^4, 10^5
500	10^3, 10^4, 10^5	10^4, 10^5, 10^6
750	10^5, 10^6, 10^6	10^5, 10^6, 10^6
1000	10^5, 10^6, 10^7	10^6, 10^6, 10^7
Mitomycin C (μM)		
0	10^2, 10^3	10^2, 10^3
0.5	$5x10^3$, 10^4	$5x10^3$, 10^4
1	10^3, 10^4	10^3, 10^4
5	10^4, 10^5	10^5, $5x10^5$
10	10^5, 10^6, 10^7	10^5, 10^6, 10^7
15	10^7	10^7
Ultraviolet light (UV) (J/m^2)		
0	10^3, $5x10^3$	10^3, $5x10^3$
10	10^5	10^5
50	10^5, $5x10^5$	10^5, $5x10^5$
100	10^5, $5x10^5$	10^5, $5x10^5$
200	$5x10^5$, 10^6	$5x10^5$, 10^6
H_2O_2 (mM)		
0	$5x10^2$, 10^3	$5x10^2$, 10^3
10	$5x10^4$	$5x10^4$
25	10^5, $5x10^5$	10^5, $5x10^5$
50	$5x10^5$, 10^6	$5x10^5$, 10^6
100	10^6, $5x10^6$	10^6, $5x10^6$

stained with methylene blue (2% in 70% ethanol) after 10-12 days in culture and counted.

2.2.7.2 UV treatment

A Stratagene Stratalinker 2400 on "dose" setting was used for UVC treatment (254 nm at a power level of 40 watts/m^2) at 10, 50, 100, or 200 J/m^2. Bulbs were preheated for two minutes before use, and run for one minute in between doses to keep them warm. ES cells were plated into 30 mm feeder plates the day before irradiation (see Table 2.2). Medium was removed from the cells immediately before irradiation, lids were removed from the plates for UV exposure, and medium plus feeders were added back to the plates immediately following irradiation (previous experience had shown it was necessary to replace the feeder cells post-irradiation as they detached from the plates following higher doses of UV). Controls were treated identically but not exposed to UV. Assays were performed in triplicate and cells were plated at two different densities per dose per replicate. Colonies were stained with methylene blue as above after ten days in culture and counted.

2.2.7.3 H_2O_2 treatment

A fresh bottle of H_2O_2 (Sigma, H0904) was used in each assay, and diluted in M-15 to 10, 25, 50, or 100 mM. ES cells were plated into 30 mm feeder plates the day before treatment (see Table 2.2). 2 ml of medium with H_2O_2 was added to each well, and plates were returned to the incubator for 15 minutes, after which the wells were washed with phosphate buffered saline (PBS), and medium and feeders were replaced (as with UV treatment, feeder cells detached after higher doses of H_2O_2). Controls were treated identically but not exposed to H_2O_2. Assays were performed in triplicate and cells were plated at two different densities per dose per replicate. Colonies were stained with methylene blue as above after ten days in culture and counted.

2.2.7.4 Mitomycin C (MMC) treatment

A freshly-thawed stock solution of MMC was diluted in M-15 to 0.5, 1, 5, 10, or 15 μM. ES cells were plated into 30 mm feeder plates the day before treatment (see Table 2.2). 3 ml of medium with MMC was added to cells, and

the plates were returned to the incubator. After four hours, plates were washed three times with PBS and fresh medium was added. Controls were treated identically but not exposed to MMC. Assays were performed in triplicate and cells were plated at two different densities per dose per replicate. Colonies were stained with methylene blue as above after 10-12 days in culture and counted.

2.3 DNA METHODS

2.3.1 Southern blotting and radioactive probes

Southern blotting was performed essentially as described (Ramirez-Solis, 1993). Random-primed probes were prepared using the Amersham RediPrime or T7 Quick-Prime kits according to the manufacture's instructions and hybridized overnight at 65°C in modified Church and Gilbert buffer (1% (w/v) bovine serum albumin (BSA; Sigma, B-4287), 1 mM ethylenediaminetetraacetic acid (EDTA), 7% (w/v) sodium dodecyl sulphate (SDS), 0.5 M $NaHPO_4$ buffer pH 7.2. 1 M $NaHPO_4$ buffer is 134 g Na_2HPO_4 and 4 ml phosphoric acid adjusted to pH 7.2 (Church and Gilbert, 1984)). Blots were washed at 65°C with SET (0.15 M NaCl, 20 mM Tris/Cl pH 7.8, 1 mM EDTA) and SDS at low (2X SET and 0.2% SDS), medium (0.2X SET and 0.1% SDS), or high (0.1X SET and 0.1% SDS) stringencies until at 10-50 counts per second, then were exposed to film.

Radiolabeled probes used for analysis were **Probe A:** 5' external *Brca1* probe, a 1.4 kb *Eco*RV/*Not*I fragment from genomic sequence upstream of exon 1. **Probe B:** internal probe, 340 bp *Bam*HI/*Nhe*I *Brca1* genomic fragment from intron 2. **Probe C:** 5' external probe, 444 bp of genomic sequence immediately upstream of *Brca1* exon 1. **Probe D:** 600 bp *Xho*I/*Xba*I *Brca1* genomic fragment from 5.5 kb downstream of exon 2. **Melk probe:** 558 bp 3' external probe amplified by PCR (primers: forward 5' aag caa acg cac cat cct ggc cac ctg 3' and reverse 5' atc ata aat gca tac ctt ggt aag ctt tc 3'. The primers and probe/digest were designed by a colleague,

Louise van der Weydn). **Gdf-9:** 5' external 650 bp probe, a *Bam*HI/*Sal*I *Gdf-9* genomic fragment containing exon1 (Dong, 1996).

2.3.2 Genotyping of Cre alleles

PCR was used to genotyping the murine Cre transgenes. 10 ng of genomic DNA was used in conjunction with Cre-specific primers (forward: 5' tcg atg caa cga gtg atg agg ttc 3'; reverse: 5' tcg cga aca tct tca ggt tct gc 3'; a gift of a colleague, Xiaozhong Wang) which generate a 172 bp product from positive samples and were a Cycling conditions were: 94°C for 5 min; 30 or 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min; 72°C for 10 min. A representative PCR is shown as an example (Figure 4.5).

2.4 RNA METHODS

2.4.1 RNA isolation

Confluent ES cells were passaged onto gelatin plates one day before harvest. Total RNA was isolated using TRIzol reagent (Gibco/Life Technologies) according to the manufacture's instructions, or with a Qiagen RNAeasy midi kit (Qiagen, Ltd), also according to instructions.

2.4.2 RT-PCR

First-strand cDNA synthesis was performed on 5 μg total RNA using a pool of anchored oligonucleotide dT primers (1/3 each $T_{18}A$, $T_{18}G$, $T_{18}C$) and SuperScript II RNaseH- Reverse Transcriptase (Invitrogen). PCR was performed using primers in exon 1 (5' ctt ggg gct tct ccg tcc tc 3') and exon 6 (5' cct tgt gct tcc ctg tag gc 3') of *Brca1*. Cycling conditions were: 94°C for 5 min; 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec; 72°C for 10'.

2.4.2.1 Semi-quantitative RT-PCR

RT-PCR was performed as above, on 5 μg of total RNA isolated from ES cells. Initial optimization experiments were carried out to determine optimal Mg^{+2} ion concentration, primer concentrations, and annealing temperatures for reactions containing either *Brca1* exon 1 and exon 6 (sequences above) or *Gapd* primers, or both sets of primers. Once these were determined, more optimization experiments were used to determine the range of cycles in which the *Brca1* and *Gapd* products were undergoing exponential amplification, using cDNA templates at a range of dilutions. In duplex PCR reactions, *Gapd* primers were added to the reactions following completion of cycle 6 because the *Gapd* product reached plateau before the *Brca1* PCR products (*Gapd* primers were also used at a lower concentration than the *Brca1* primers for the same reason). Under these conditions, neither product had reached plateau by 33 cycles.

Total cDNA was diluted 1:40, 1:80, or 1:160 in distilled water, and 1 μl was used in a 25 μl PCR reaction containing 1x buffer (supplied), 2 mM $MgCl_2$, 0.2 μmol of each *Brca1* primer and 0.06 μmol of each *Gapd* primer, and 1 unit of Platinum Taq DNA Polymerase (Invitrogen – to ensure "hot-start" PCR). PCR (95°C for 5 min; 31 or 33 cycles of 95°C for 30 sec, 59°C for 45 sec, 72°C for 1 min; 72°C for 10 min) was run for 31 or 33 cycles; earlier optimization experiments indicated that at these cycle numbers, neither product had yet reached the plateau stage. 12 μl of each reaction was loaded onto 2% agarose gels and photographed using the Eagle Eye system (Stratagene). Band volumes were quantitated using Image Quant software (Molecular Dynamics). *Brca1* band volume for each lane was normalized to the corresponding *Gapd* volume. Two RNA samples were used for each genotype, and each experiment was run in triplicate. Mean and standard deviations were calculated in Microsoft Excel for each genotype based on all normalized replicate values.

2.4.3 5' RACE

5' Rapid Amplification of cDNA Ends (5' RACE) was performed using a 5' RACE system (Invitrogen), according to the manufacturer's instructions. Briefly, 5 μg total RNA was used as a template for reverse transcription using a gene specific primer (GSP; *Brca1* X.10R7: 5' gct tct tga ggg gcg gtc tg 3') in conjunction with SuperScriptII RNaseH⁻. Resulting products were tailed using terminal deoxynucleotidyl transferase in conjunction with dCTP.

First-round PCR of tailed 5' RACE products was performed using an Abridged Anchor Primer (AAP; 5' ggc cac gcg tcg act agt acg ggi igg gii ggg iig 3'; "i" represents inosine) which recognizes the C-tailed product, in conjunction with a nested, *Brca1* reverse primer. Nested primers used were: *Brca1* X.5R: 5' gga cat tgt gaa ggc cct ttc 3'; *Brca1* X.7R: 5' caa ggt ggc att tcc agg ttc 3'; *Brca*1 X.8R2 5' cac tga tct cac gat tcc aag g 3'; and *Brca1* X10R: 5' cag acc gcc cct caa gaa gc 3'.

2.4.3.1 Sequencing of 5' RACE products

PCR products were isolated from agarose gels using the Qiagen Gel Extraction Kit (Qiagen Ltd.) and cloned into the pGEM T-Easy vector (Promega), in which Sp6 and T7 bacterial promoter sequences flank the multiple-cloning site. Sequencing was performed using T7 (5' gta ata cga ctc act ata ggg c 3') or Sp6 (5' att tag gtg aca cta tag aa 3') primers and BigDye chemistry (PE Biosystems). Cycling conditions were: 94°C for 4 min; 35 cycles of 94°C for 20 sec, 50°C for 5 sec, 60°C for 4 min. Reactions were cleaned by centrifugation through G-50 Sephadex mini spin-columns (Corning/Costar Spin-X, 0.45 μM filter) for 2 minutes at 4000 rpm.

2.4.3.2 Direct sequencing of 5' RACE products

10 l of 5' RACE PCR product was mixed with 2 units each of Exonuclease I and Shrimp Alkaline Phosphatase (Roche) to rid the reaction of leftover primers, and incubated for one hour at 37°C, then enzymes were inactivated at 90°C for 15 minutes. The reaction was desalted on a Micron YM-100

column (in accordance with the manufacturer's instructions regarding DNA clean-up) before sequencing with a nested, reverse *Brca1* primer (specific primers are listed in figure legends, primer sequences are given in section 2.4.3).

2.4.4 Northern blot analysis and radiolabeled probes

20 μg of total RNA was run on 0.9% agarose denaturing formaldehyde gels in 0.5x MOPS buffer (20 mM MOPS (3-(N-Morpholino)propanesulfonic acid), 2 mM sodium acetate, 1 mM EDTA). RNA was blotted onto Hybond-N nylon membrane (Amersham Biosciences) and fixed by UV crosslinking in a Stratalinker (Stratagene) on the auto-crosslink setting. Prior to hybridization, blots were washed in 150 mM NaP, 0.1% (w/v) SDS buffer at 60°C for an hour (NaP buffer is 0.5 M Na_2HPO_4 with 3.9 ml H_3PO_4 per litre). Probes were generated as in section 2.3.1. Blots were both pre-hybridized (10 minutes) and hybridized (overnight) at 60°C in hybridization buffer (15% (v/v) formamide, 7% (w/v) SDS, 0.01% (w/v) BSA (Sigma, A-3803), 0.35 M NaP buffer. 1 mg freshly-boiled ssDNA was added to both prehybridization and hybridization reactions). Blots were washed in 150 mM NaP, 0.1% (w/v) SDS buffer at 55°C until at 10-50 counts per second, then exposed to film. Blots were also exposed to a PhosphorImager screen and scanned on a Typhoon 8600 scanner (Molecular Dynamics). Blots were serially re-hybridized (after stripping) as indicated in (Figure 6.8). Band intensity and relative transcript amounts were determined using ImageQuant analysis software (Molecular Dynamics). All transcripts were measured in relation to the expression of a *Gapd* loading control.

Most probes used for analysis were generated by PCR amplification of AB2.2 wildtype ES cell cDNA (cycling conditions: 95°C 5 min; 35 cycles of 95°C for 30 sec, 55°C for 45 sec, 72°C for 1 min; 72°C for 10 min). Primers and probes: ***Brca1* 3' end probe:** (forward: 5' ctg tgt ggg gct tcc gtg gt 3' and reverse: 5' gga gtc ttg tgg ctc act ac 3'; 250 bp probe), ***Nbr1*:** (forward: 5' ggt aga aaa cca agc ggc tg 3' and reverse: 5' cct ctg aaa tag gca ttg ag 3'; 314 bp

probe), ***Gapd* (*glyceraldehyde-3-phosphate dehydrogenase*):** (forward: 5' gca aat tca acg gca cag tc 3' and reverse: 5' cag agg ggc cat cca cag tc 3'; 420 bp probe), and ***p21*:** (forward: 5' ggt ggt ggg ggt ggg ctt atc 3', reverse: 5' gct ttg ggg tcg ggt gtg agg 3'; 376 bp probe). Other probes used were a ***Bard1* cDNA probe** (a 400 bp *Eco*RI/*Xho*I 5' *Bard1* cDNA fragment from the *Bard1* expression construct described in section 2.1.1.11), and a **PGK promoter probe** (the first 250 bp of the promoter, excised with *Eco*RI and *Spe*I from pPGK-*Puro*-bpA).

2.5 PROTEIN TECHNIQUES

2.5.1 Immunolocalization of Brca1 in embryoid bodies

This work was performed by Dr. Michal Goldberg in the lab of Dr. Steve Jackson, Wellcome CRC laboratory, Cambridge, England. Differentiated ES cells were passaged at high density onto poly-L-lysine (Sigma) coated glass coverslips and grown for one day. Cells were fixed in ice-cold methanol for 15 minutes on ice followed by permeabilization in ice-cold acetone for 20 seconds. Coverslips were washed with PBS and blocked in 10% FBS (v/v) in PBS, then incubated with a 1:4 dilution of primary antibody (Brca1 M-20, an affinity-purified goat polyclonal antibody raised to amino acids 1793-1812 of the mouse protein, from Santa Cruz Biotechnology) in 5% FBS (v/v) in PBS. Cells were washed with PBS, and fluorescein isothiocyanate (FITC)-conjugated anti-goat secondary antibody (Jackson Laboratories) were added. Coverslips were washed again with PBS and mounted with Vectashield mounting medium containing propidium iodide (Vector Labs) or TOTO-3 iodide (Molecular Probes, T-3604). Slides were viewed with a BioRad confocal laser microscope by sequential scanning of the two emission channels used (488 nm for FITC, 514 nm for propidium iodide and rhodamine, 660 nm for TOTO-3 iodide).

2.5.1.1 Immunolocalization of Brca1 following DNA damage

Cells were grown as above. Prior to fixation, cells were either treated with 50 J/m^2 UVC in a Stratalinker (Stratagene), or γ-irradiated with 1500 rads. In

both cases, cells were fixed four hours after damage, then stained and scanned as above.

2.5.2 Extraction of protein from cells

ES cells used for protein extraction were passaged onto gelatin plates when confluent (to clear feeder contamination) and grown until confluent again. ES cells or Phoenix cells were washed with PBS and incubated for 5' on ice in CelLytic M mammalian cell lysis buffer (Sigma) with protease inhibitors added (Sigma P8340; mammalian mix, used at a 1:100 dilution. This mix contains 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin). Lysates were collected by scraping, and debris centrifuged out. Total protein concentration was determined by Bradford assay (BioRad) using bovine serum albumin (New England Biolabs) as a control protein.

2.5.3 Antibodies

Brca1: **GH118** mouse monoclonal antibody was a gift of Shridar Ganesan of the Dana-Farber Cancer Institute, Boston, Massachusetts. This antibody was raised against a GST-fusion of the C-terminal third of mouse Brca1. **M-20** is an affinity-purified goat polyclonal antibody raised against Brca1 residues 1793-1812 (Santa Cruz Biotechnology). **GST**: Rabbit affinity-purified polyclonal antibody (Santa Cruz Biotechnology; sc-459). **c-myc**: Mouse monoclonal IgG_1 raised to residues 408-439 of human p62 c-Myc (Clontech). **α-tubulin:** Mouse monoclonal antibody (IgG^1) from Sigma (F-2168) used at 1:1000 dilution. **Secondary antibodies:** alkaline-phosphatase conjugated goat-anti-rabbit (A-0418) or goat-anti-mouse (A-3562) antibodies were purchased from Sigma. A 1:5000 dilution of secondary antibody in 10 ml block solution was routinely used. For enhanced chemi-luminescence (ECL) detection, horseradish-peroxidase conjugated anti-mouse antibody was purchased from Pierce and used at a 1:5000 dilution.

2.5.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

For routine Western analysis of protein, lysates comprising 30 μg of total protein were mixed with an equal volume of 2x sample buffer (0.14 M Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.01% (w/v) bromophenol blue, and 5 mM freshly-added dithiothreitol), boiled for 10 min, and separated on polyacrylamide gels containing SDS (SDS-PAGE gels) (Laemmli, 1970). Proteins were electroblotted onto polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Bedford, MA). Membranes were blocked with 1.5% (w/v) BSA (Sigma, B-4287) in TBST (25 mM Tris-HCl pH 7.6, 125 mM NaCl, 0.1% (v/v) Tween 20). Primary and secondary antibodies were diluted in this blocking solution. Membranes were incubated with rocking, then washed three times for 10 minutes with TBST. Secondary antibodies (described in section 2.5.3) were diluted in blocking solution and added to the membrane as above. After incubation, membranes were washed again, 3 times for 15 minutes in TBST.

Conjugated secondary antibodies were detected using BCIP/NBT (5-Bromo-4-Chloro-3-Indolyl phosphate/nitro blue tetrazolium) Sigma FAST tablets. This colourimetric reaction was stopped by rinsing the blot in 25 mM EDTA. Blots were digitally scanned using an Epson1640SU desktop scanner. For ECL analysis, following the wash step, blots were incubated in ECL working solution (Pierce) for 5 minutes and exposed to film for 1-5 minutes to generate images. Films were scanned into the computer.

2.5.4.1 SDS-PAGE analysis and Western blotting of Brca1

Duplicate aliquots of 30 μg total protein from ES cell lysates were subjected to SDS-PAGE analysis on simultaneously-run 7% polyacrylamide gels, as above. GH118 (1:5 dilution in 5 ml block solution) was used as the primary antibody on one, α-tubulin (diluted 1:10000) on the other. Horseradish-peroxidase conjugated anti-mouse (diluted 1:5000) was used as the secondary antibody on both.

2.5.5 Co-immunoprecipitation/pulldown of Brca1 and Bard1 fusion proteins

Cell lysates from Phoenix cells transfected with GST-Brca1 and/or myc-Bard1 fusion proteins vectors containing 1 mg total protein were used for GST or c-myc immunoprecipitations. Lysates were adjusted to 500 μl using CelLytic lysis buffer in 1.7 ml tubes. GST pulldown: 40 μl GST beads (glutathione-agarose (Sigma, G4510), 50% slurry in lysis buffer) were added to lysates. Tubes were incubated at 4°C on a rotating wheel overnight. c-myc immunoprecipitation: 3 or 4 μl c-myc antibody (24 or 32 μg) was added to each lysate. Tubes were incubated at 4°C on a rotating wheel for three hours, then 40 μl protein G beads (Protein G on agarose (Sigma P7700), 50% slurry) were added. Rotation at 4°C continued overnight.

Beads were collected by centrifugation and washed at normal (three washes in cold CelLytic lysis buffer with a 1:1000 dilution of mammalian protease inhibitor cocktail), or stringent (three washes in radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 0.01% (v/v) IGPAL, 0.01% (w/v) SDS, 50 mM Tris pH 8.0, 1 mM EDTA) with a 1:1000 dilution of mammalian protease inhibitor cocktail, then three washes with PBS) conditions. An equal volume of 2x sample buffer was added to washed beads, samples were boiled for ten minutes, and 15 μl of each sample was subjected to electrophoresis on a 10% SDS-PAGE gel. Western blotting was performed as described in section 2.5.4. Primary antibodies were: c-myc (1:500 dilution in 10 ml block solution) or GST (1:1000 dilution in 10 ml block solution); secondary antibodies were anti-rabbit (GST) or anti-mouse (c-myc).

2.6 MICE

2.6.1 Blastocyst injection of targeted ES cells

2.6.1.1 Germline transmission of the Brca1⁻ allele

Two representative *+/–* ES cell lines (clones C2 and D2) were injected into blastocysts as described (for an overview, see (Ramirez-Solis, 1993)). Both successfully underwent germline transmission as confirmed by Southern blot analysis of *Hind*III-digested tail-tip genomic DNA using the 5' external probe A, as described above (Table 2.1/Figure 3.1). Subsequent genotyping of mice was by Southern blot analysis of *Hind*III-digested genomic DNA using probe B (see Table 2.1).

2.6.1.2 Germline transmission of the c1 allele

Two representative *–/c1* ES cell lines (clones D9 and F2) were injected into blastocysts. One (F2) successfully underwent germline transmission, confirmed by Southern blot (see Table 2.1).

2.6.1.3 Blastocyst injection of –/gol and +/gol ES cells

Blastocyst injections using *–/gol* ES cells generated only low-percentage chimæras which did not transmit the allele through the germline, likely because these cells underwent a number of manipulations in culture. After generation of the *Brca1*-gollum-TV vector and successful targeting of this vector into wildtype cells, one representative *+/gol* ES cell clones was injected (H8); germline transmission of this allele has not yet been achieved.

2.6.2 Viability of –/– mice

Heterozygous intercrosses (*Brca1*$^{+/-}$ X *Brca1*$^{+/-}$) were used to generate animals for a tumourigenesis study. Progeny of heterozygous intercrosses were screened for the *Brca1*$^{-}$ allele by Southern analysis of *Hind*III-digested genomic DNA using probe B.

2.6.3 Tumourigenesis studies/Screening of mammary glands

2.6.3.1 Brca1$^{+/-}$; Blm$^{-/-}$ tumourigenesis studies in Houston and England

Blm mice were generated by Guangbin Luo; the allele used in this study was *Blm^{Brdm3}* (Luo, 2000). The *Blm* allele was genotyped by Southern analysis of *Bam*HI-digested genomic DNA using a 1.5 kb *Sal*I/*Kpn*I genomic probe. The probe recognizes fragments of 6.5 kb (wildtype) or 5.7 kb (targeted) (Figure 4.1). *Brca1$^{+/-}$* mice were crossed to *Blm$^{-/-}$* mice, and the progeny were intercrossed to generate control *Brca1$^{+/-}$; Blm$^{+/-}$* and experimental *Brca1$^{+/-}$; Blm$^{-/-}$* mice.

Initially, thirty mated and thirty virgin females for both *Brca1$^{+/-}$; Blm$^{+/-}$* and *Brca1$^{+/-}$; Blm$^{-/-}$* genotypes were to be generated for this study. However, a flood at the Baylor College of Medicine mouse facility in June of 2001 removed many of the mice from this study. A second study was started in England to age 57 female and 50 male *Brca1$^{+/-}$; Blm$^{-/-}$* mice.

2.6.3.1.1 Whole Mount analysis of mammary glands from Brca1$^{+/-}$ mice: Dr. Daniel Medina from Baylor College of Medicine performed whole-mount analysis of the mammary glands of several mice. Five *Brca1$^{+/-}$; Blm$^{+/-}$* or *Brca1$^{+/-}$; Blm$^{-/-}$* female mice, aged 15-16 months and seven aged 22-24 months were analyzed (Figure 4.2).

2.6.3.2 Tumourigenesis study using mice carrying the c1 allele

Mice carrying the *c1* allele were crossed to mice carrying one of two Cre transgenes: the ubiquitously expressed CMV-Cre, a knock-in at the *Hprt* locus on the X-chromosome (generated by colleagues Hong Su and Xiaozhong Wang (Su, 2002)), or a Cre transgene knocked in at the *β-casein* locus (generated by colleagues Guangbin Luo and Yue He). Mice carrying one of the Cre transgenes and *c1* in various combinations have been aged in a tumourigenesis study (Table 4.1).

2.7 ANALYSIS SOFTWARE

Lasergene software (DNASTAR Inc., Madison, Wisconsin, USA) was used for sequence analyses. Searches for PEST sequences were done using PESTfind (Rogers); relative stability of Brca1 and Brca1^{gol} proteins was assessed using ProtParam (Bioinformatics1, 2003). Alignments were generated using Lasergene software, ClustalW (Thompson, 1994), and ESPript (Gouet, 1999). Most graphs and statistical analyses were performed using Microsoft Excel, except the Kaplan-Meier plots in Chapter 4 (Figures 4.4 and 4.6), which were generated using SigmaStat (SSPS, Inc.).

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).

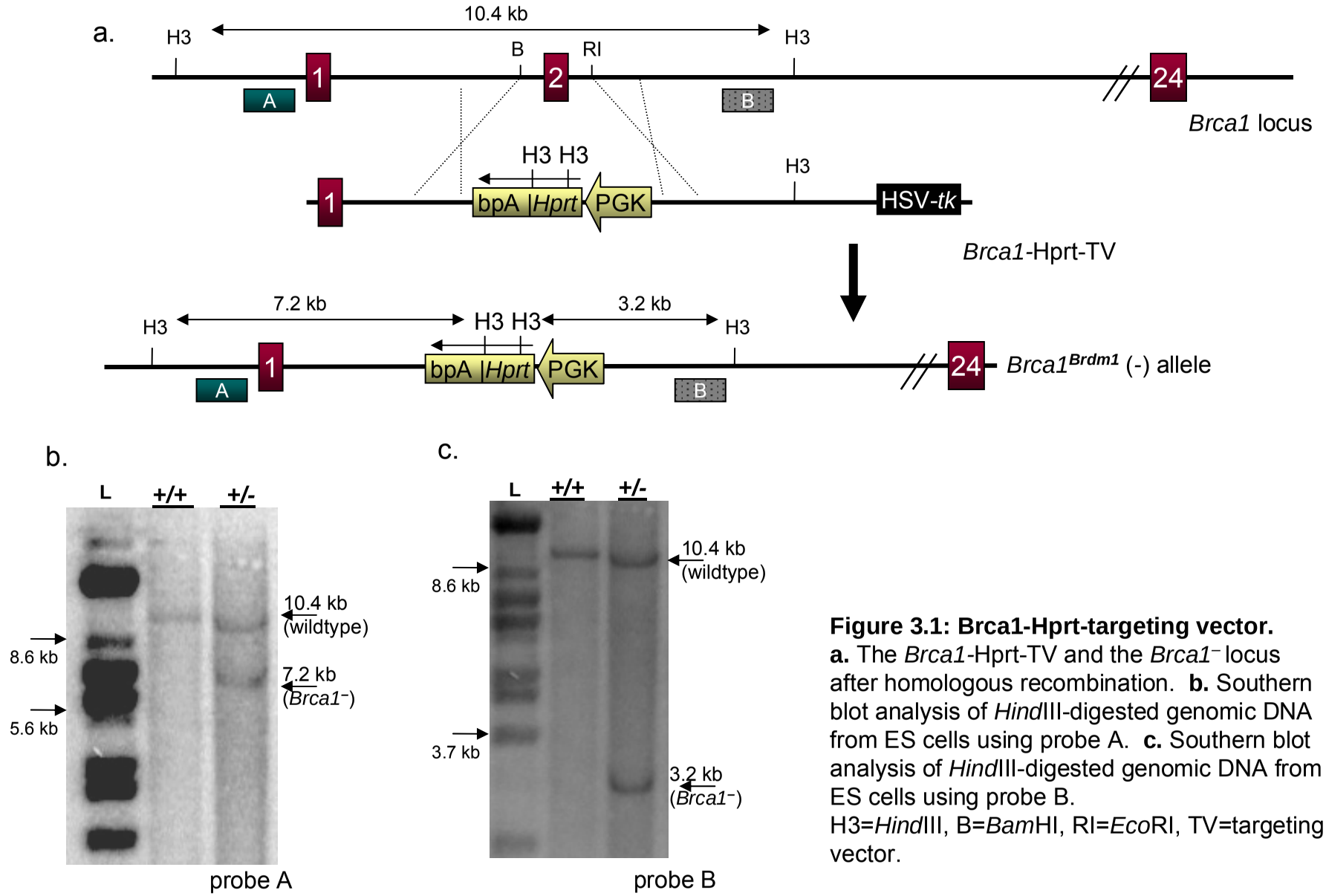


Figure 3.1: Brca1-Hprt-targeting vector. **a.** The *Brca1*-Hprt-TV and the *Brca1*$^{-}$ locus after homologous recombination. **b.** Southern blot analysis of *Hind*III-digested genomic DNA from ES cells using probe A. **c.** Southern blot analysis of *Hind*III-digested genomic DNA from ES cells using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

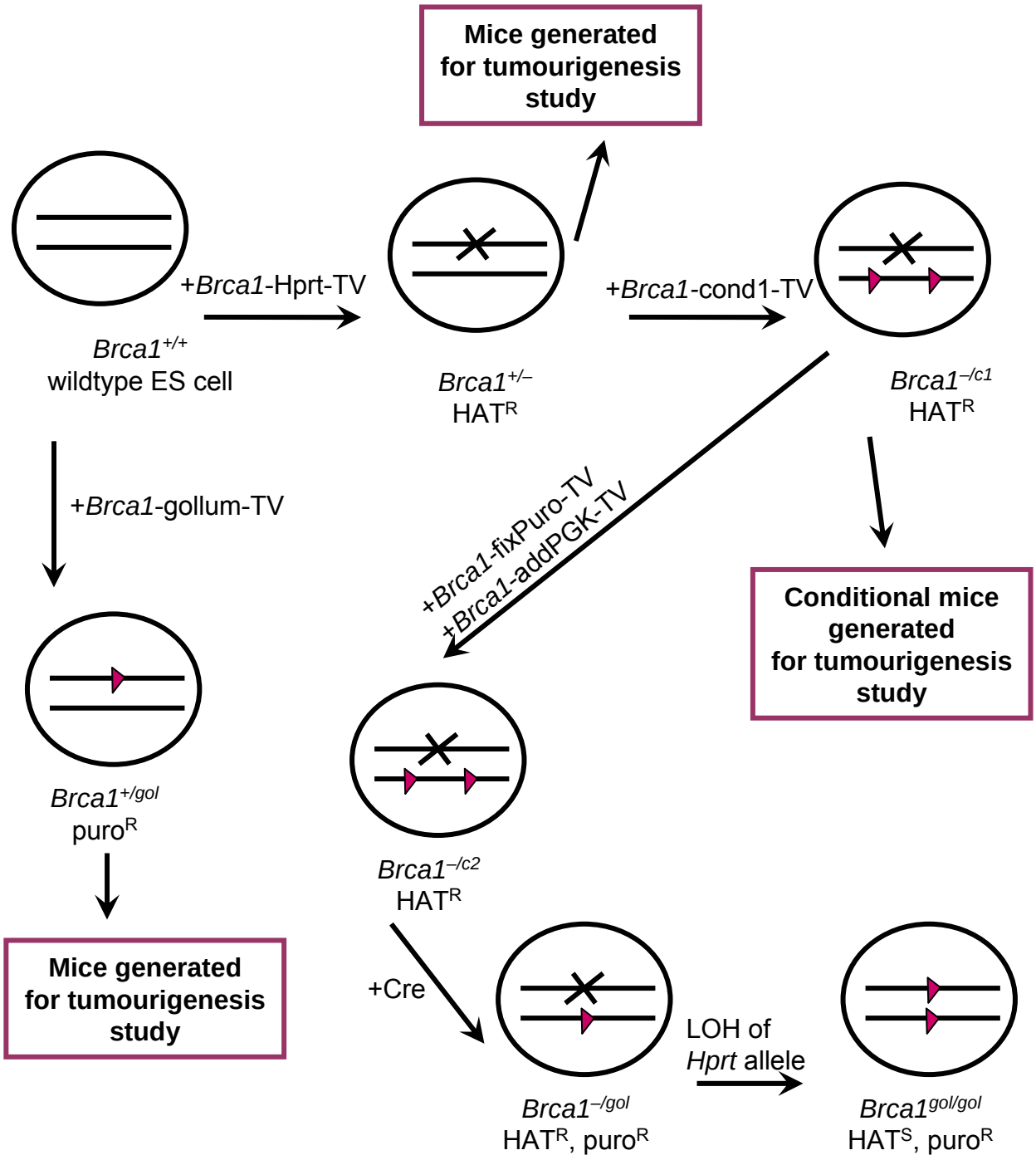


Figure 3.2: Overview of targeting events and generation of ES cells for blastocyst injection. This schematic shows the various targeting events described in this work. First, *Brca1*-Hprt-TV was electroporated into wildtype ES cells. These cells were used to generate mice and also for targeting of the *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.

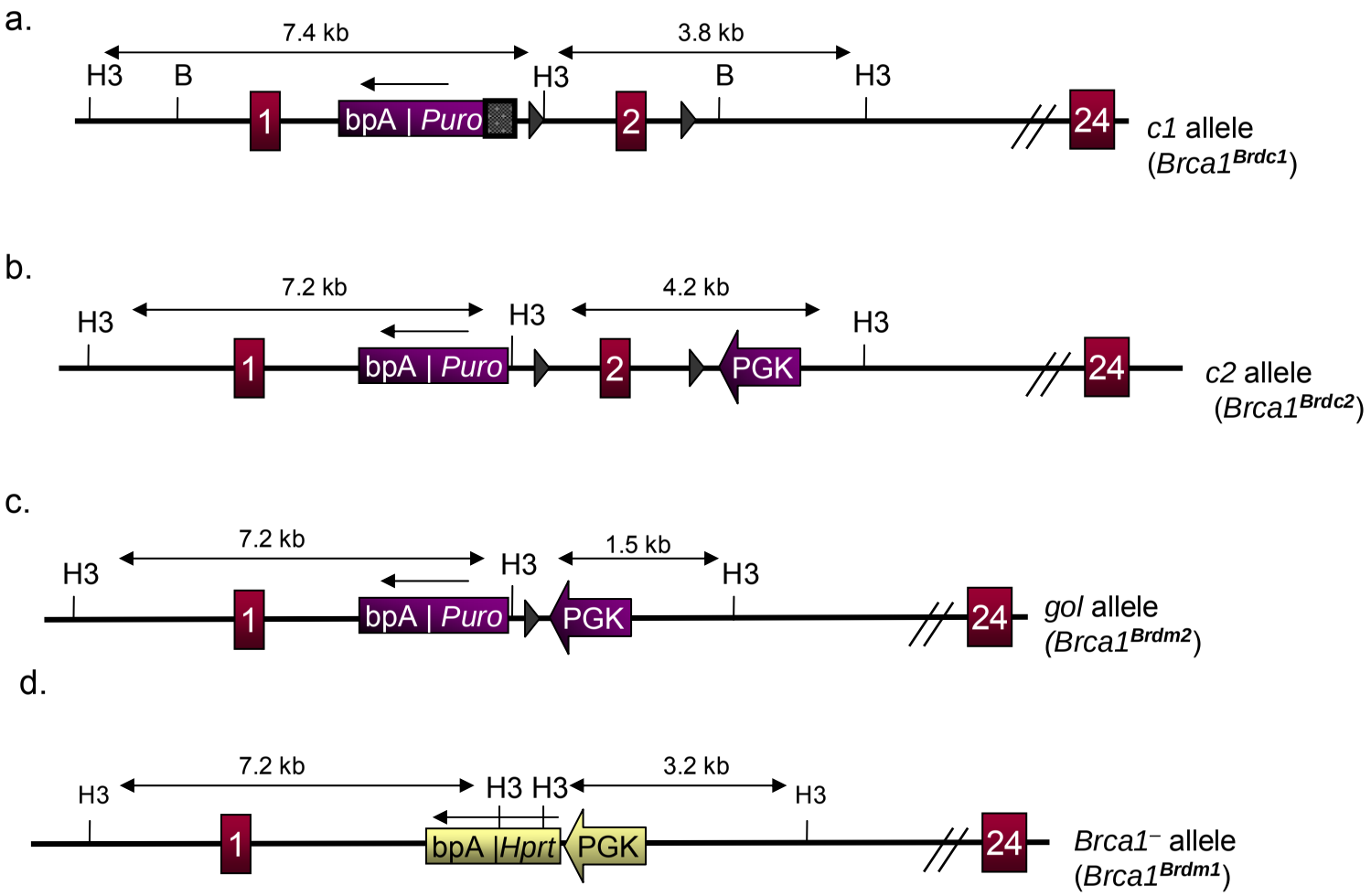


Figure 3.3: Overview: the alleles generated in this study.

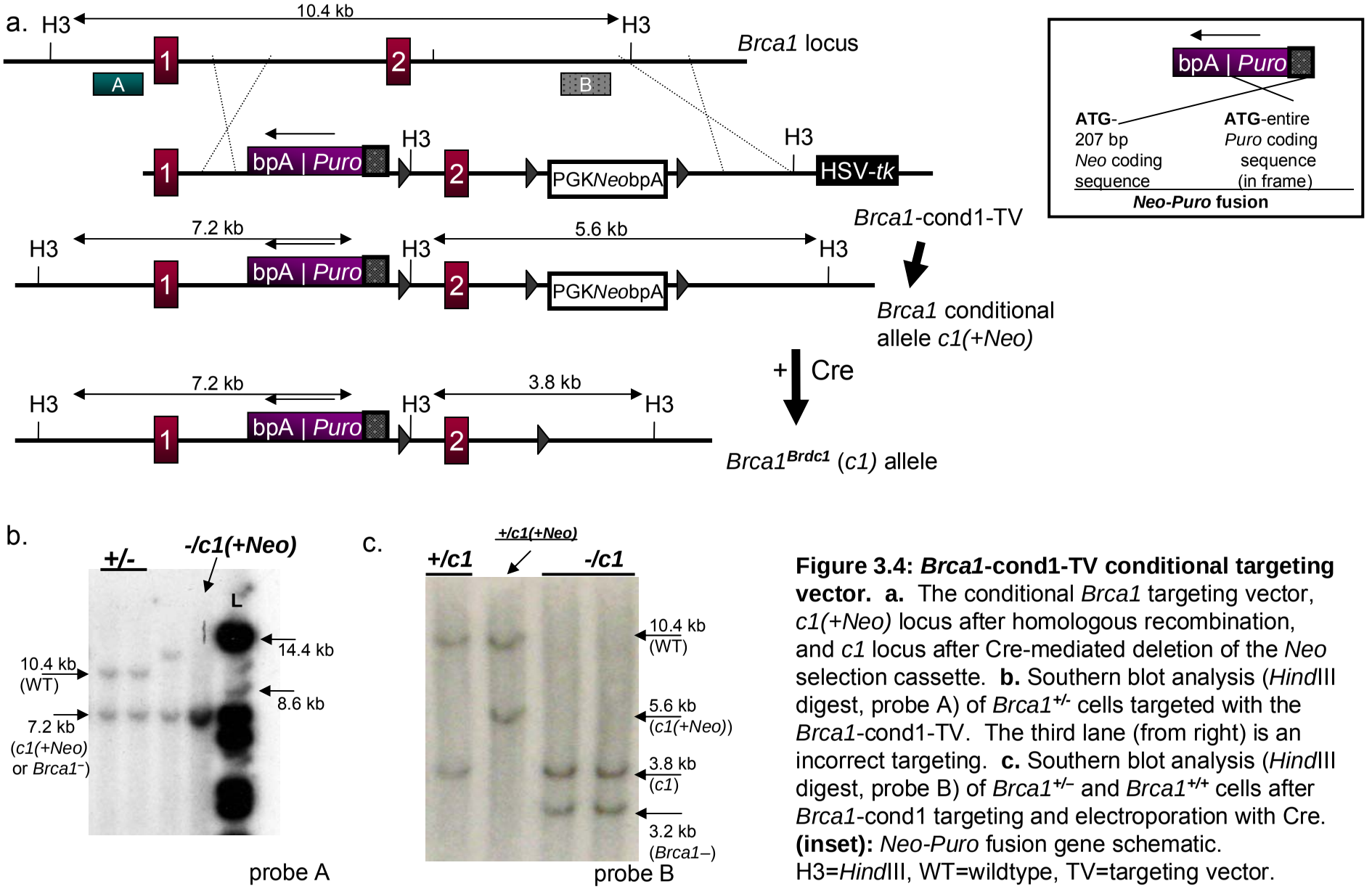


Figure 3.4: *Brca1*-cond1-TV conditional targeting vector. a. The conditional *Brca1* targeting vector, *c1(+Neo)* locus after homologous recombination, and *c1* locus after Cre-mediated deletion of the *Neo* selection cassette. **b.** Southern blot analysis (*Hind*III digest, probe A) of $Brca1^{+/-}$ 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

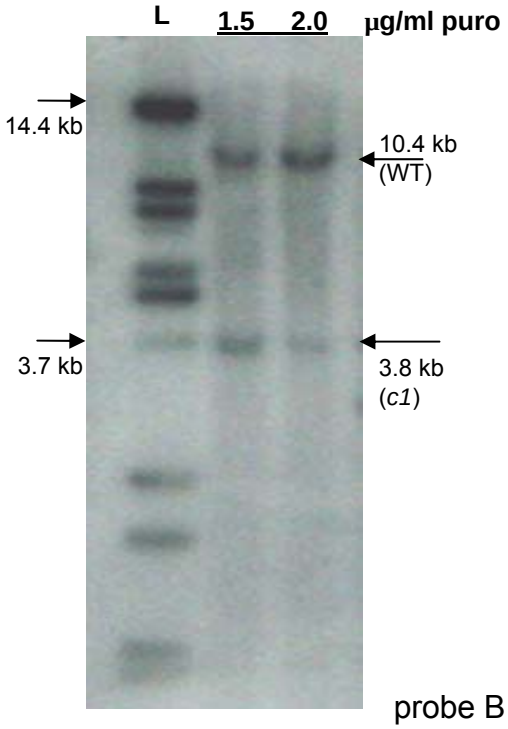


Figure 3.5: The *c1* allele is not efficiently excised by low doses of puro. *+/c1* ES cell colonies selected in 1.5 or 2 μg/ml puro were analyzed by Southern blot (*Hind*III digest, probe B). The *c1* allele (3.8 kb) is slightly less prevalent in cells selected at 2.0 μg/ml, but the background of un-excised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

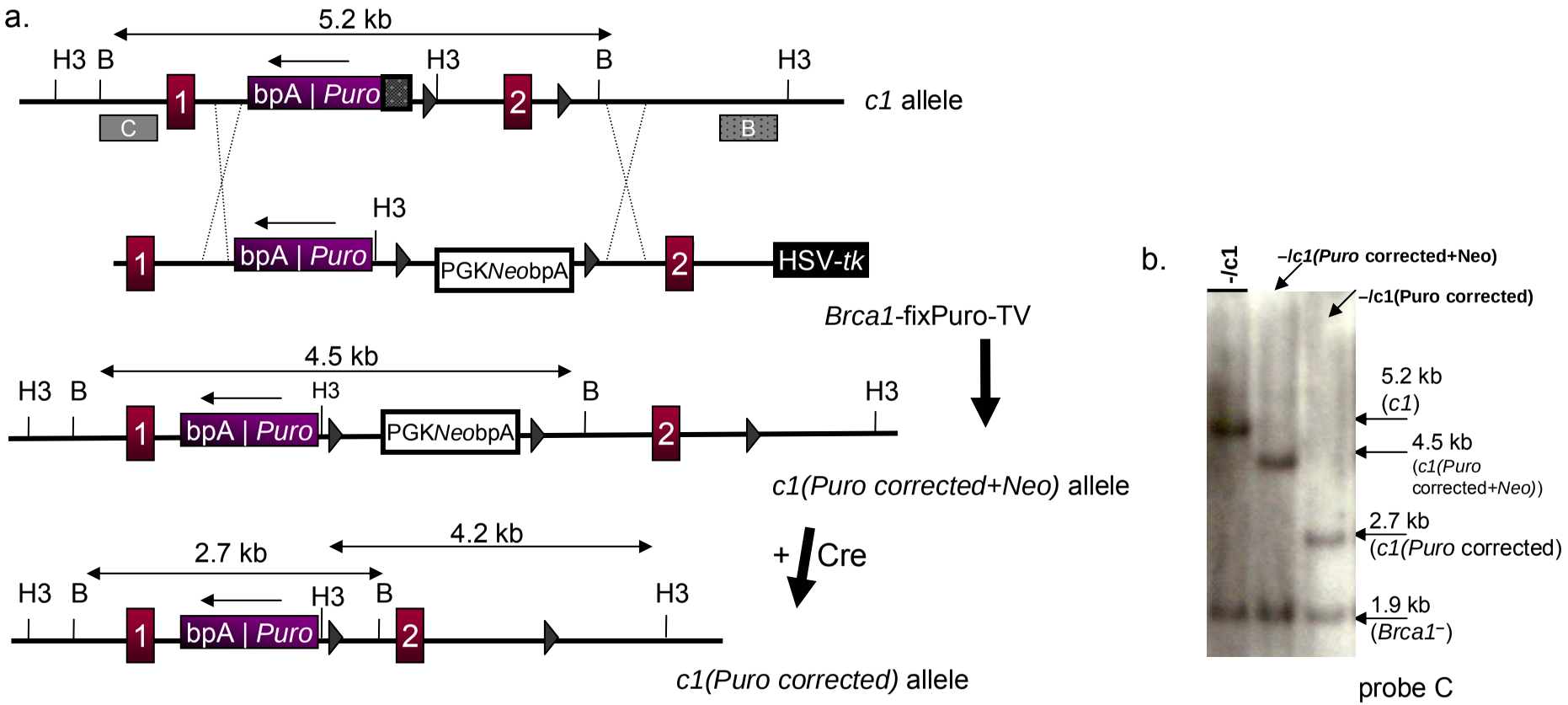


Figure 3.6: *Brca1*-fixPuro targeting vector. a. The *c1* allele and the *Brca1*-fixPuro-TV. After integration and Cre-mediated excision, the mutated *Neo-Puro* gene is replaced. **b.** Southern blot analysis (*Bam*HI digest, probe C) of *–/c1* cells after targeting and Cre-mediated excision of the selection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

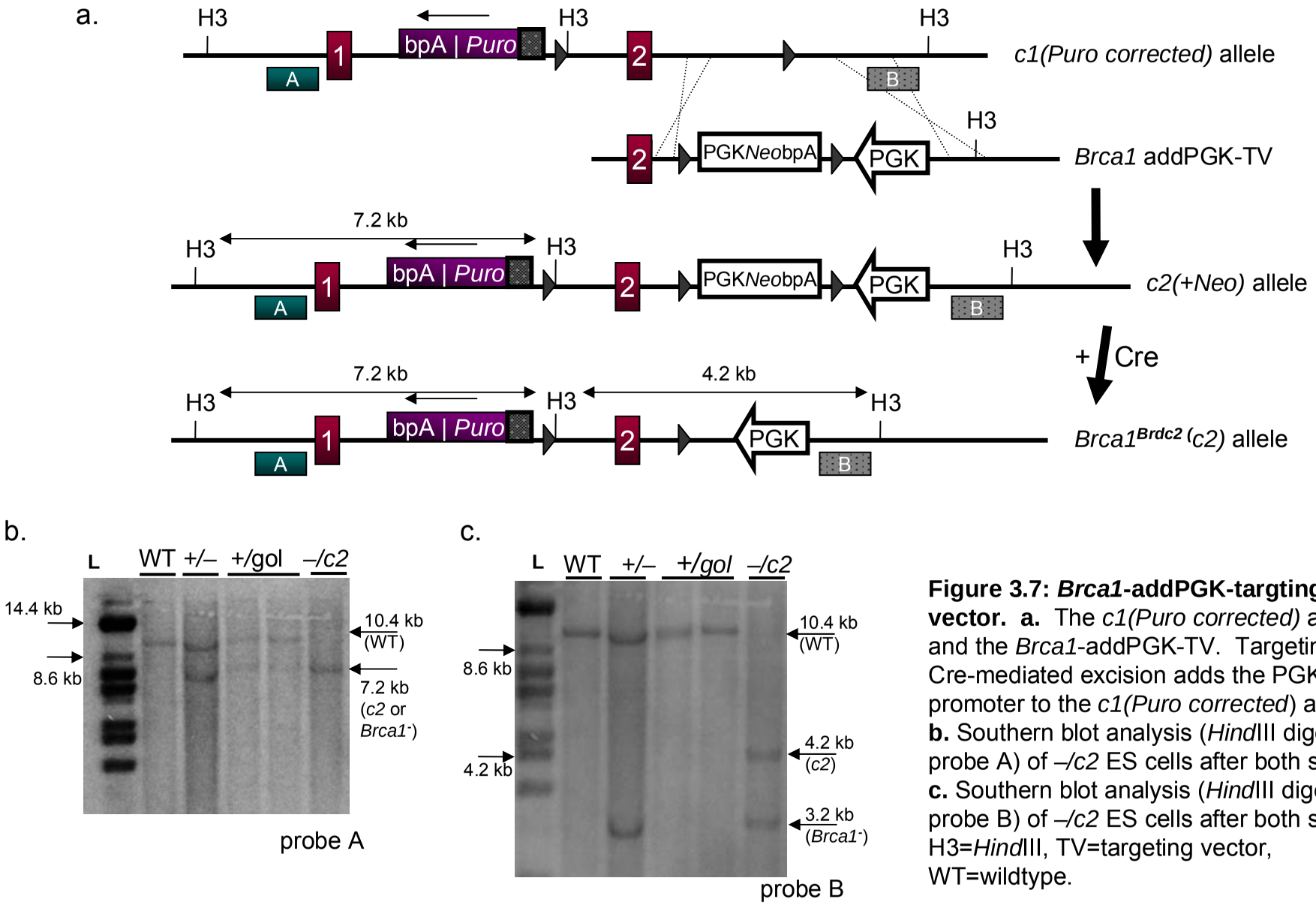


Figure 3.7: *Brca1*-addPGK-targting vector. a. The *c1(Puro corrected)* allele and the *Brca1*-addPGK-TV. Targeting and Cre-mediated excision adds the PGK promoter to the *c1(Puro corrected*) allele. **b.** Southern blot analysis (*Hind*III digest, probe A) of *–/c2* ES cells after both steps. **c.**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

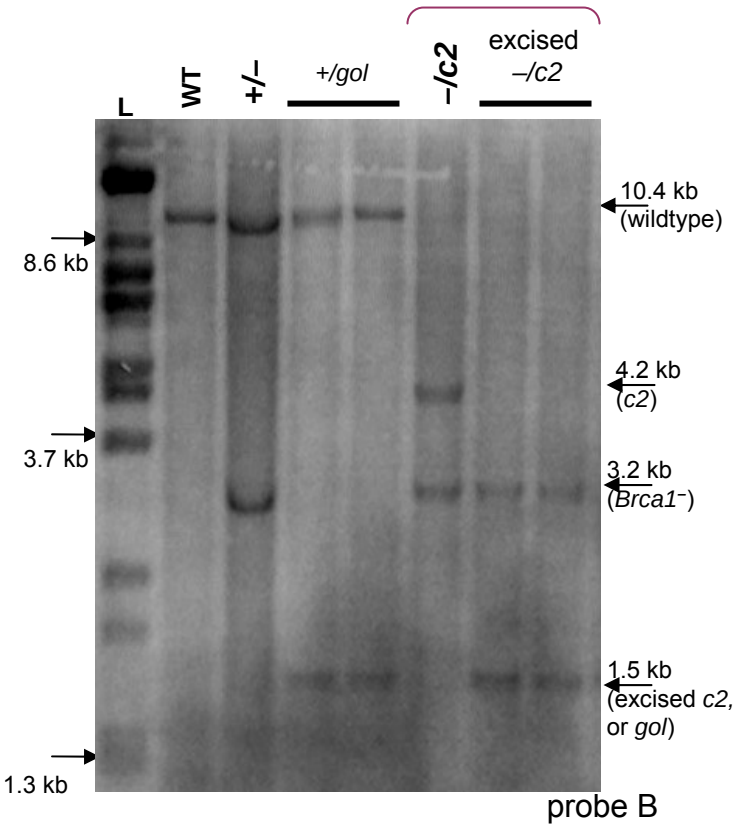


Figure 3.8: *–/c2* ES cells after Cre-mediated excision. Southern blot analysis (*Hind*III digest, probe B) of puro-resistant colonies resulting from Cre-mediated excision of the *c2* allele from *–/c2* ES cells. Genomic DNA from the parental *–/c2* ES cells is shown as a reference. WT=wildtype.

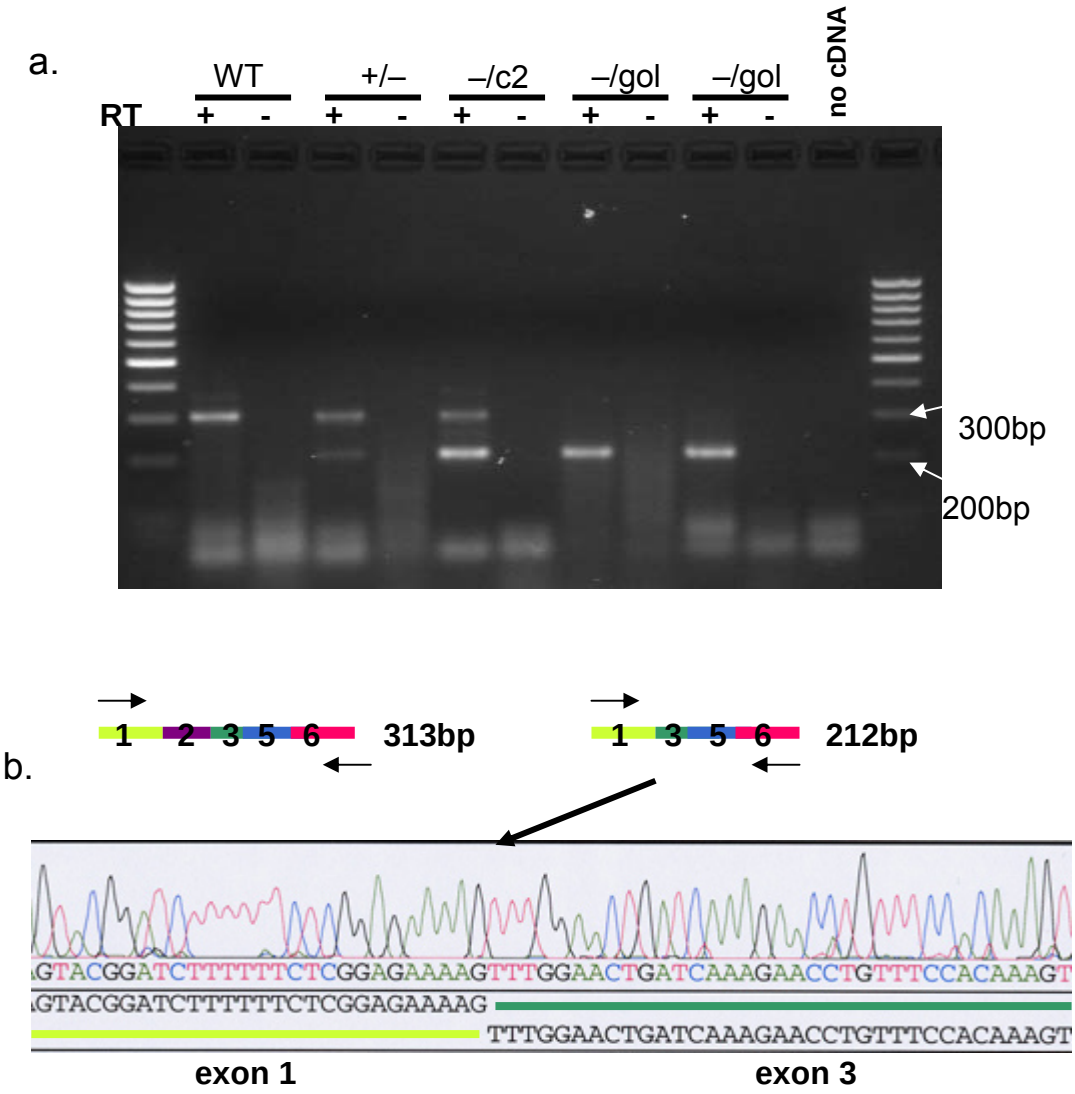


Figure 3.9: –/gol cells lack full-length *Brca1* mRNA. a. RT-PCT of wildtype, +/–, and *–/gol* cells reveals that while no full-length *Brca1* mRNA is produced (313 bp), a exon1-3 splice isoform (212 bp) is expressed in *–/gol* and +/– cells. **b.** Sequence analysis of the smaller product reveals a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

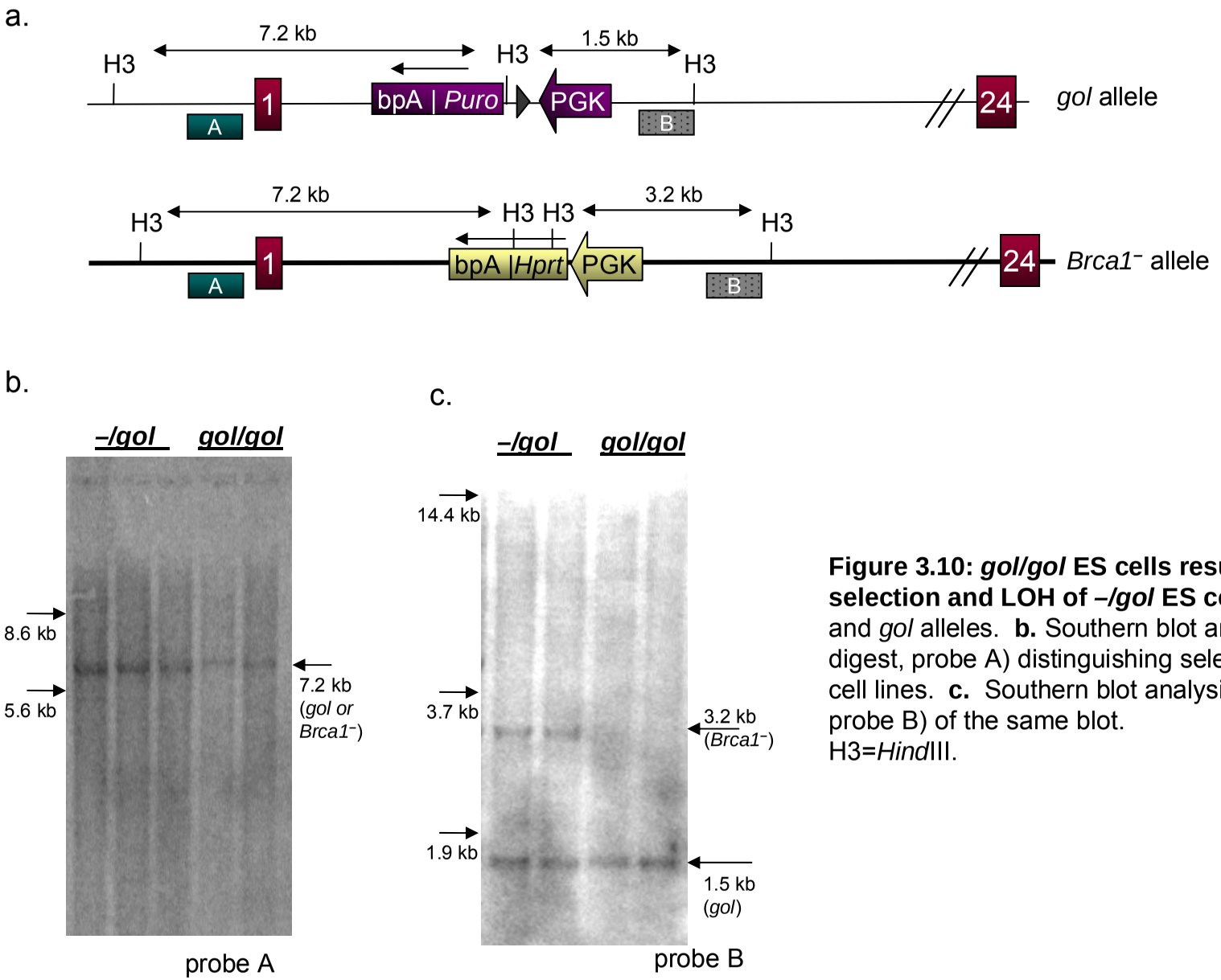
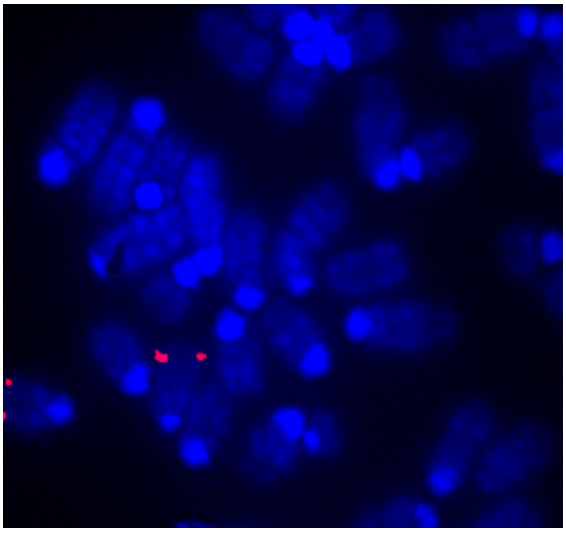
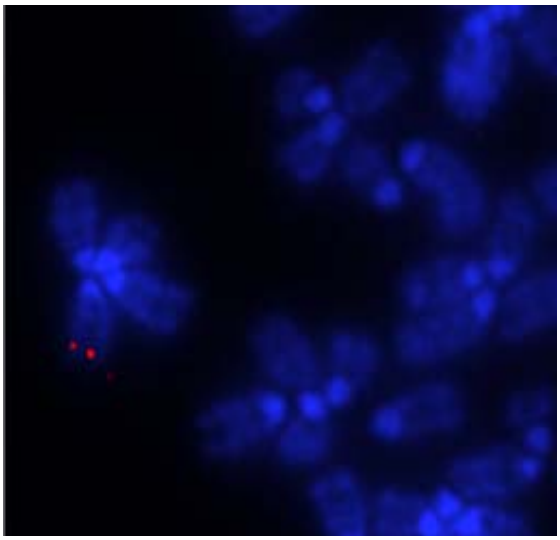


Figure 3.10: *gol/gol* ES cells resulting from 6TG selection and LOH of *–/gol* ES cells. a. *Brca1*⁻ and *gol* alleles. **b.** Southern blot analysis (*Hind*III digest, probe A) distinguishing selected and parental cell lines. **c.** Southern blot analysis (*Hind*III digest, probe B) of the same blot.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

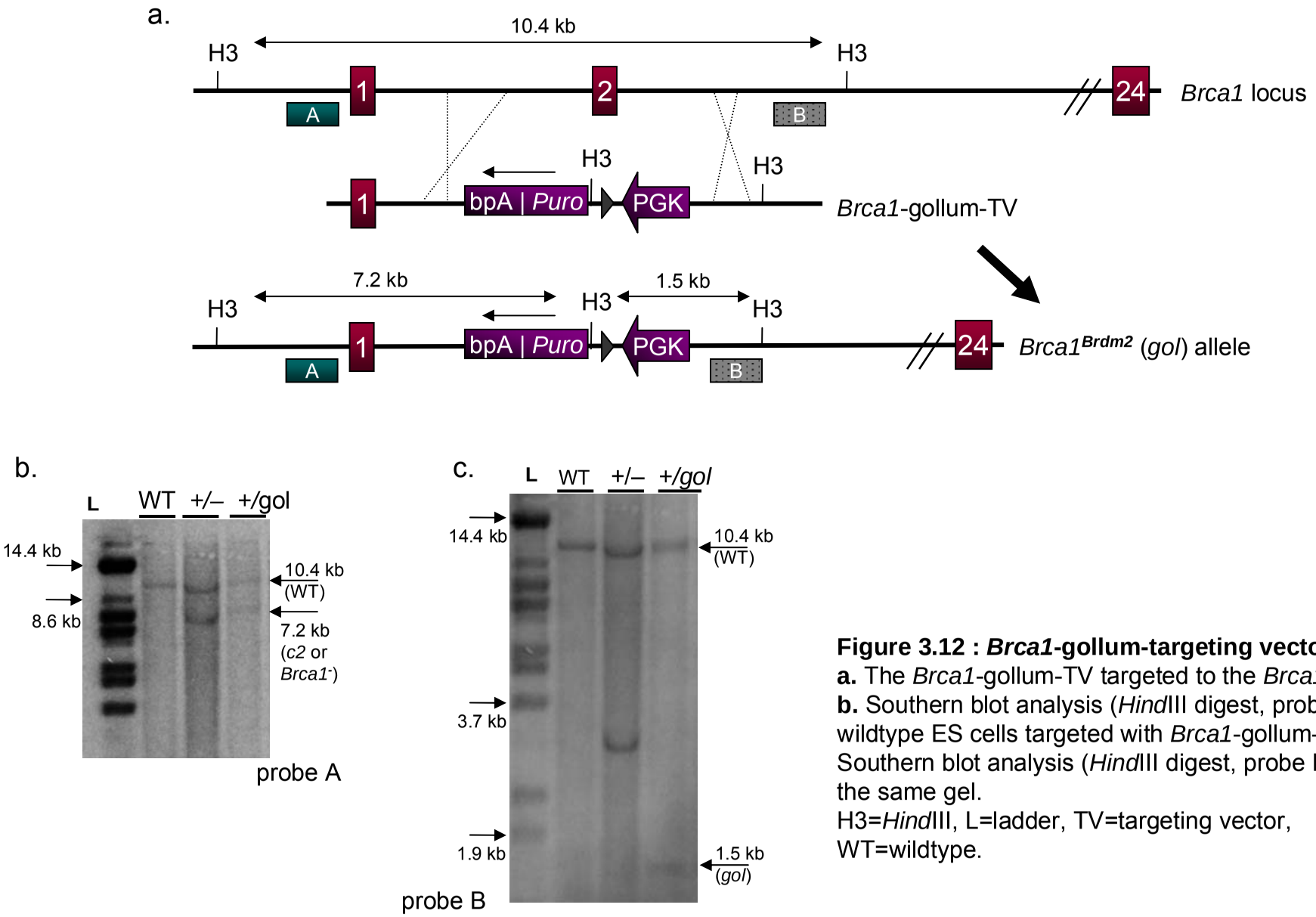


Figure 3.12 : *Brca1*-gollum-targeting vector.
a. The *Brca1*-gollum-TV targeted to the *Brca1* locus. **b.** Southern blot analysis (*Hind*III digest, probe A) of wildtype ES cells targeted with *Brca1*-gollum-TV. **c.** Southern blot analysis (*Hind*III digest, probe B) of the same gel.
H3=*Hind*III,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

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)*

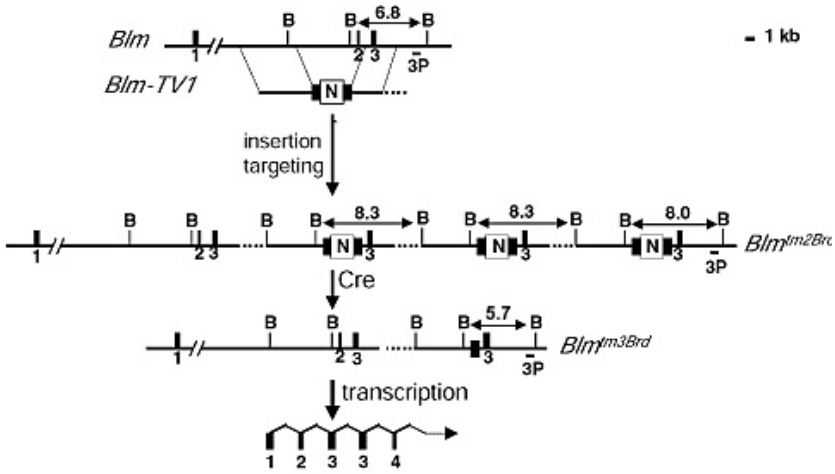
Earlier studies have indicated that mice carrying a *Brca1* knockout allele do not have an increased predisposition to tumourigenesis compared to wildtype mice. Several groups have used secondary mutations to try and accelerate *Brca1*–related tumourigenesis, most commonly by crossing the mutation onto 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.



b.

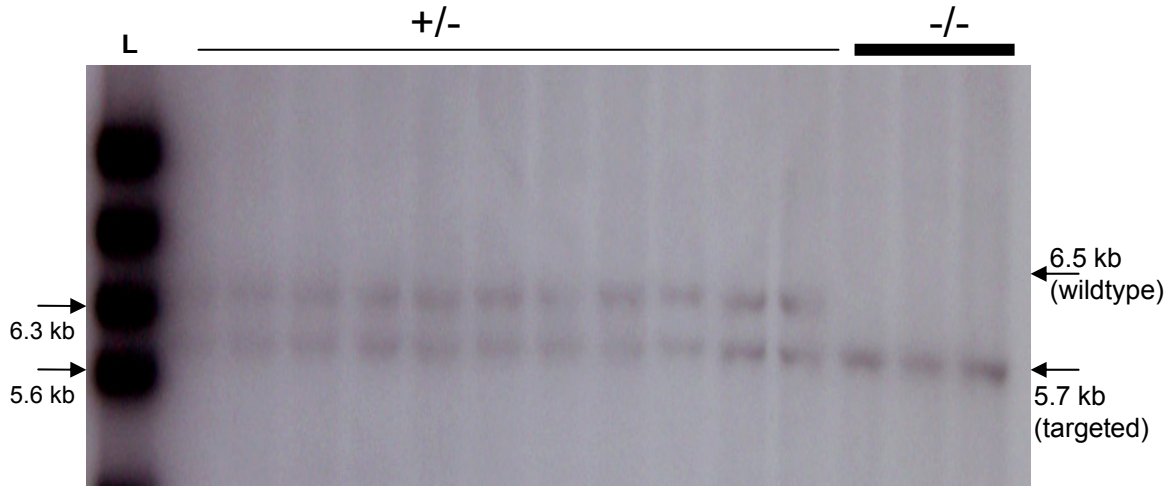


Figure 4.1: *Blm* locus genotyping. a. Blm targeting vector schematic. **b.** Southern blot analysis of *Bam*HI-digested mouse genomic DNA probed with the *Blm* genotyping probe.
L=ladder. Targeting vector figure modified from (Luo, 2000).

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.

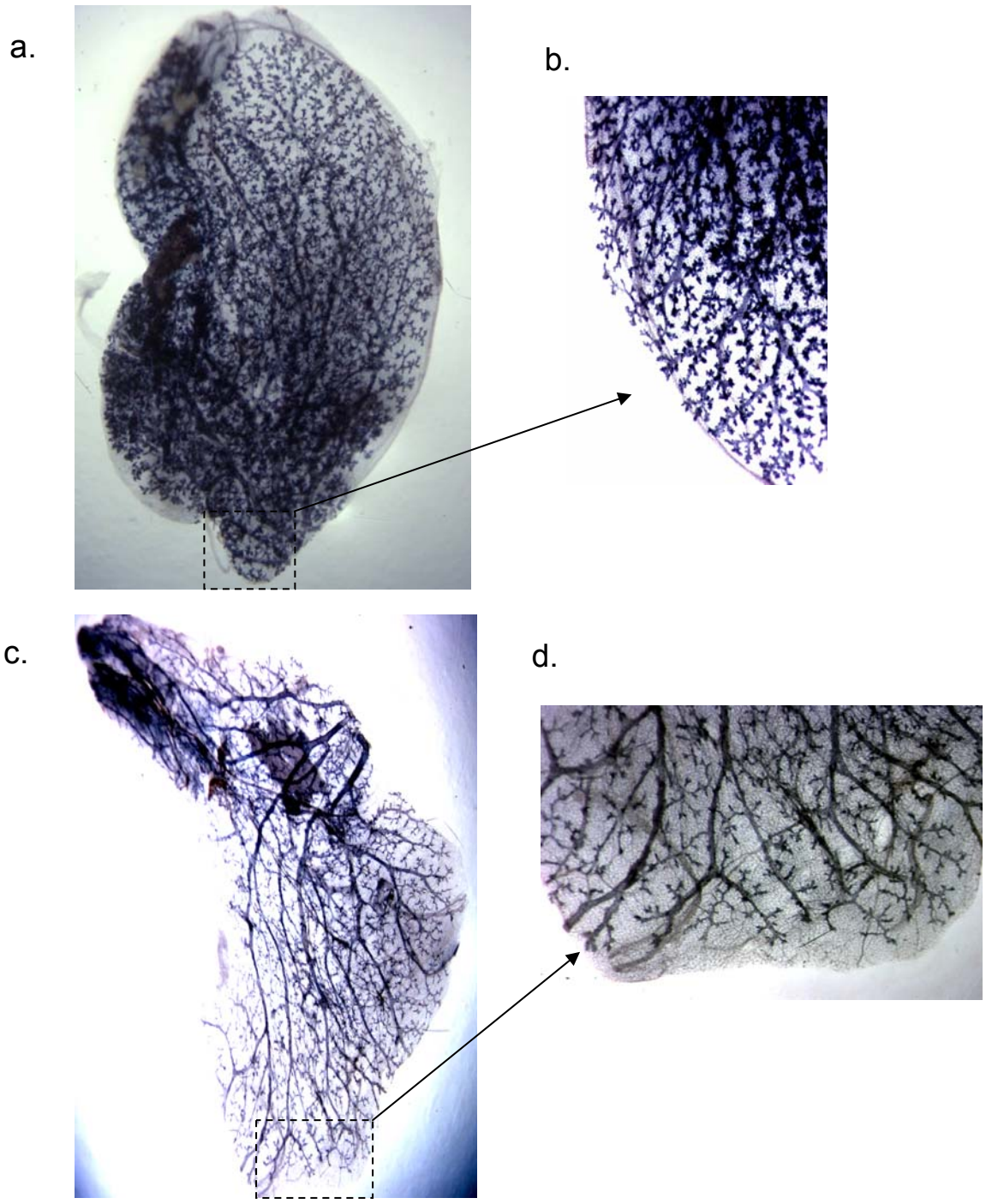


Figure 4.2: Whole-mount analysis of mammary glands from *Brca1*$^{+/-}$*; Blm*$^{-/-}$ *or Brca1*$^{+/-}$*; Blm*$^{+/-}$ mice. **a.** A mammary gland from a 16 month old, female *Brca1*$^{+/-}$*, Blm*$^{-/-}$ mouse. **b.** Same sample, close-up to show normal branching structure of ductal tree. **c.**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

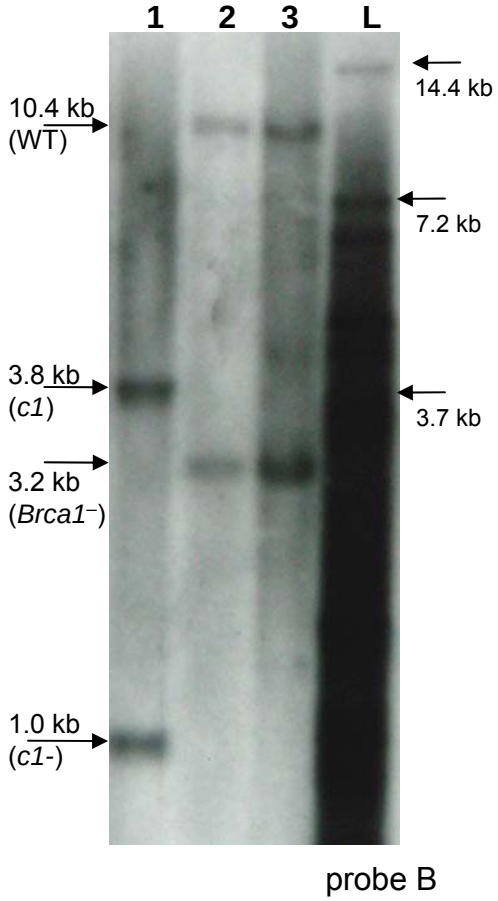


Figure 4.3: Tumours have not lost the wildtype allele of *Brca1*.
Southern blot analysis (*Hind*III digest, probe B) of tumours from a *c1/c1*– mouse (lane 1), and two tumours from *Brca1*$^{+/-}$, *Blm*$^{-/-}$ mice (lanes 2 and 3).
L=ladder, WT=wildtype.

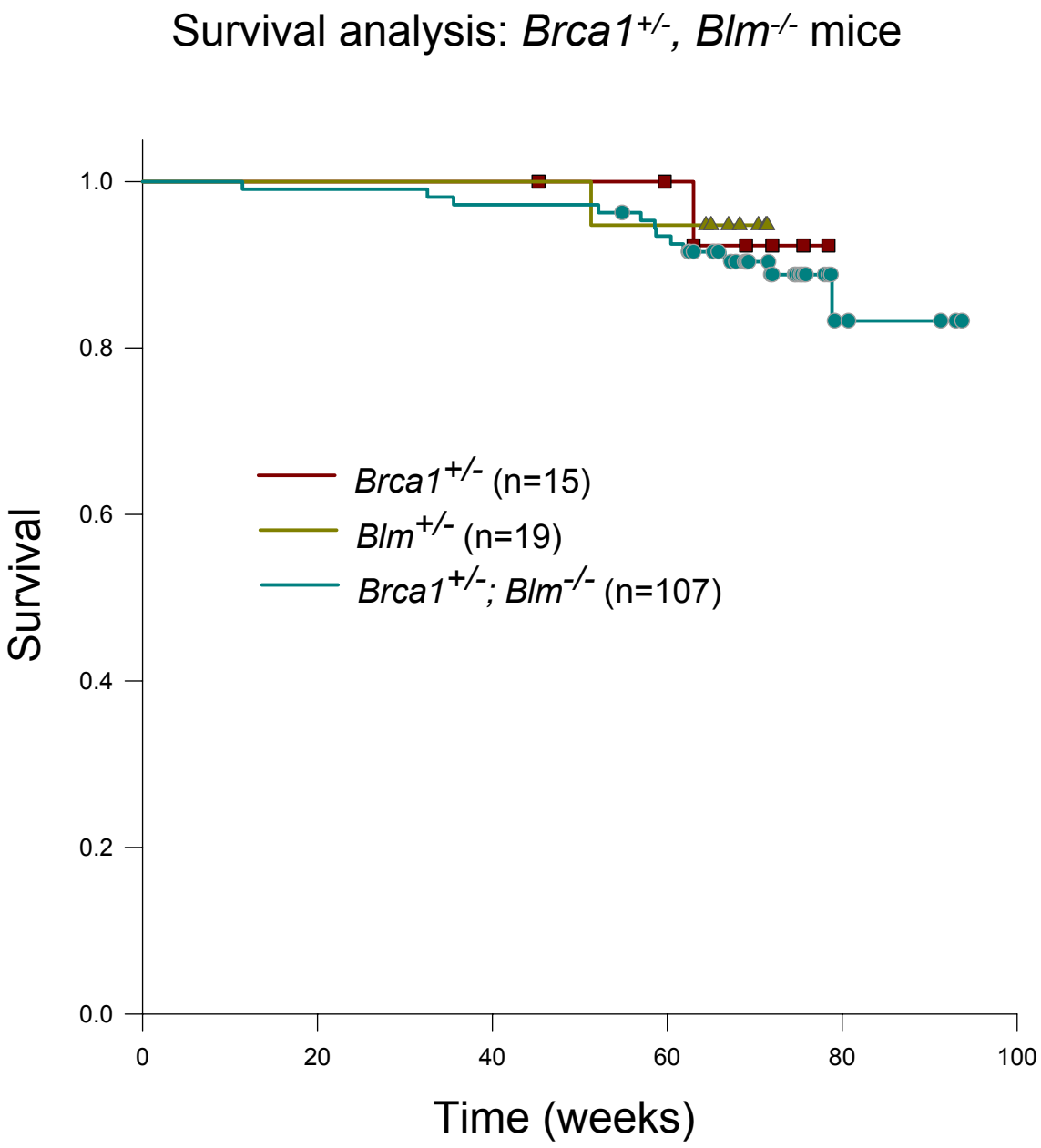


Figure 4.4: Survival analysis of *Brca1*$^{+/-}$, *Blm*$^{-/-}$ mice. Kaplan-Meier survival analysis of *Brca1*$^{+/-}$, *Blm*$^{-/-}$ and *Brca1*$^{+/-}$ and *Blm*$^{+/-}$ mice. Mice dying of natural causes, or terminated because of illness or tumourigenesis were all included. Only three *Brca1*$^{+/-}$,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

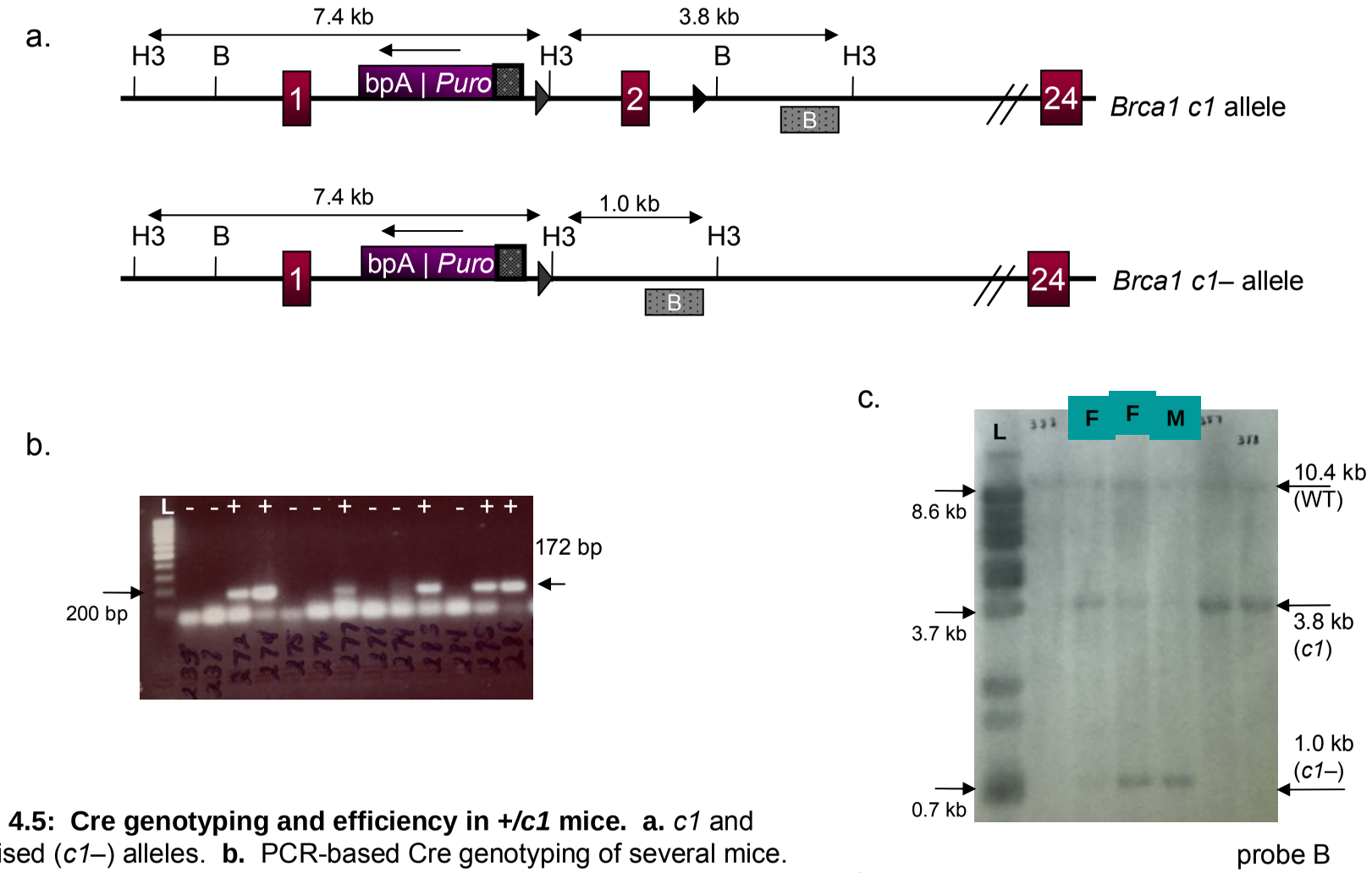


Figure 4.5: Cre genotyping and efficiency in +/*c1* mice. **a.** *c1* and *c1* excised (*c1*–) alleles. **b.** PCR-based Cre genotyping of several mice. A 172 bp product ("+") indicates the presence of Cre. **c.** *β-casein*-Cre, *Brca1*$^{+/c1}$ mice. Southern blot analysis (*Hind*III digest, probe 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

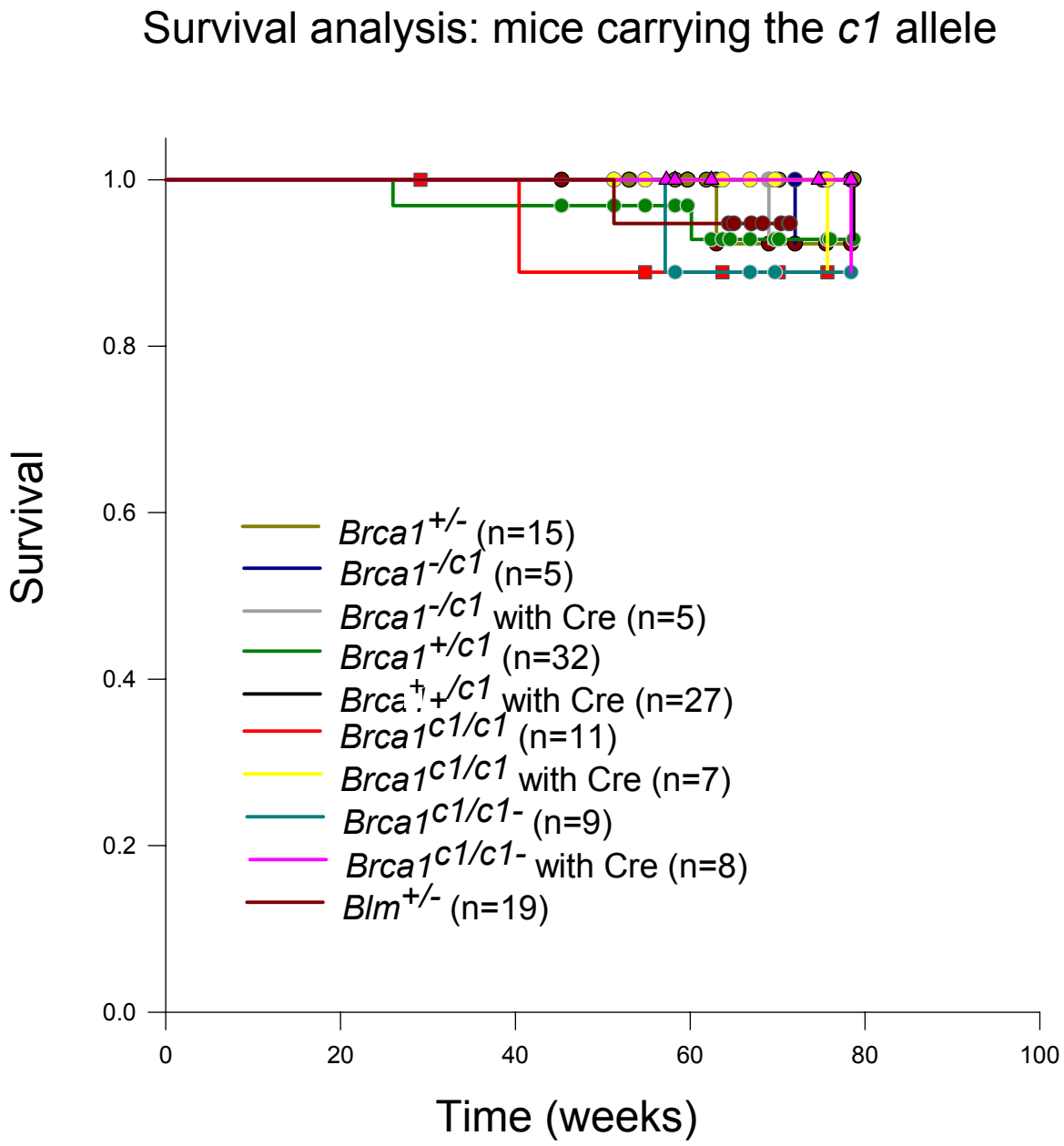


Figure 4.6: Survival analysis of mice carrying the *c1* allele. Kaplan-Meier survival analysis of mice carrying the *c1* allele with or without a Cre transgene. "Cre" in this figure means either CMV-Cre or *β-casein*-Cre; survival times for mice carrying these two transgenes did not differ. *Brca1*$^{+/-}$, *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.

CHAPTER FIVE:
THE RESPONSE OF *gol/gol* CELLS TO DNA DAMAGE

5.1 INTRODUCTION

Numerous studies have demonstrated that *BRCA1* mutations in both mouse and human cells confer hypersensitivity to DNA damage (reviewed in Kerr and Ashworth, 2001; Deng and Wang, 2003). Previous studies of the role of BRCA1 in DNA damage have not generally attempted to designate the specific domains required for resistance to a damaging agent, and in many cases, cell lines with known secondary mutations (*e.g.* tumour-derived cell lines) have been used to demonstrate sensitivity. Various damaging agents have been used in such assays; one common one is γ-irradiation. Most reports agree that the entire BRCA1 protein appears to be necessary for a normal response to this mutagen; cells homozygous for deletions of exon 11, or for deletions of the N- or C-termini are all hypersensitive to γ-irradiation (Shen, 1998; Abbott, 1999; Ruffner, 2001; Zhou, 2003). The second main goal of this project was to determine the sensitivity of *gol/gol* ES cells to various DNA damaging agents. As the *gol* allele is predicted to give rise to a protein lacking the N-terminal RING domain (referred to here as $Brca1^{gol}$), these experiments may help uncover the importance of the N-terminus in the response of Brca1 to DNA damage.

5.1.1 Mutagenic agents used in these experiments

gol/gol ES cells were exposed to four mutagenic agents: γ-irradiation (primary lesion: double-strand breaks), mitomycin C (MMC; primary lesion: interstrand cross-links), UV irradiation (primary lesion: mutated bases), and H_2O_2 (oxidative damage resulting in mutated bases). The methods used to repair such lesions were discussed in sections 1.11.3 and 1.11.4. The experiments described in this chapter indicate that *gol/gol* ES cells are not hypersensitive to mutagens which cause base damage, but do have a defect in double-strand break repair (DSBR). Once this result was obtained, the efficiencies of both homologous recombinational repair (HRR) and non-homologous end joining (NHEJ), the two major forms of DSBR, were additionally tested in *gol/gol* cells.

5.1.2 Immunolocalization of Brca1^{gol}

BRCA1 is a nuclear protein which is hyperphosphorylated and forms nuclear foci following DNA damage (Scully, 1996). Although the precise function of these foci is unknown, they contain several other proteins known to be involved in DNA repair, such as RAD51, BRCA2, BLM, and RAD50, and are likely to be involved in the sensing and/or repair of DNA damage (see section 1.11.3 for a summary) (Scully, 1997c; Chen, 1998; Wang, 2000b). It is currently unknown which domains of BRCA1 are required for damage-induced focus formation, although human HCC1937 cells, which carry a C-terminal truncated version of BRCA1, do not exhibit nuclear foci after DNA damage, while MEFs carrying only the ΔX.11 isoform of Brca1 do form damage-induced foci (Zhong, 1999; Wu, 2000; Huber, 2001).

In this study, the cellular localization of the Brca1^{gol} protein, which is thought to lack all or most of the N-terminal RING domain, was determined by immunolocalization using an antibody raised against an epitope from the C-terminus of the mouse protein. The immunolocalization patterns of Brca1^{gol} and wildtype Brca1 are very similar; both are observed in the nucleus and cytoplasm, and nuclear foci are seen in undamaged cells of both genotypes. However, the amount of cytoplasmic localization is higher than has generally been observed, and the overall localization pattern is fairly punctate. Following UV exposure, to which the *gol/gol* cells are not hypersensitive, damage-induced nuclear foci are observed in wildtype and *gol/gol* cells. Damage-induced nuclear foci are also observed following treatment with γ-irradiation, to which *gol/gol* cells are hypersensitive, but a proportion of γ-irradiated *gol/gol* cells also appear to have large aggregates of Brca1^{gol} protein.

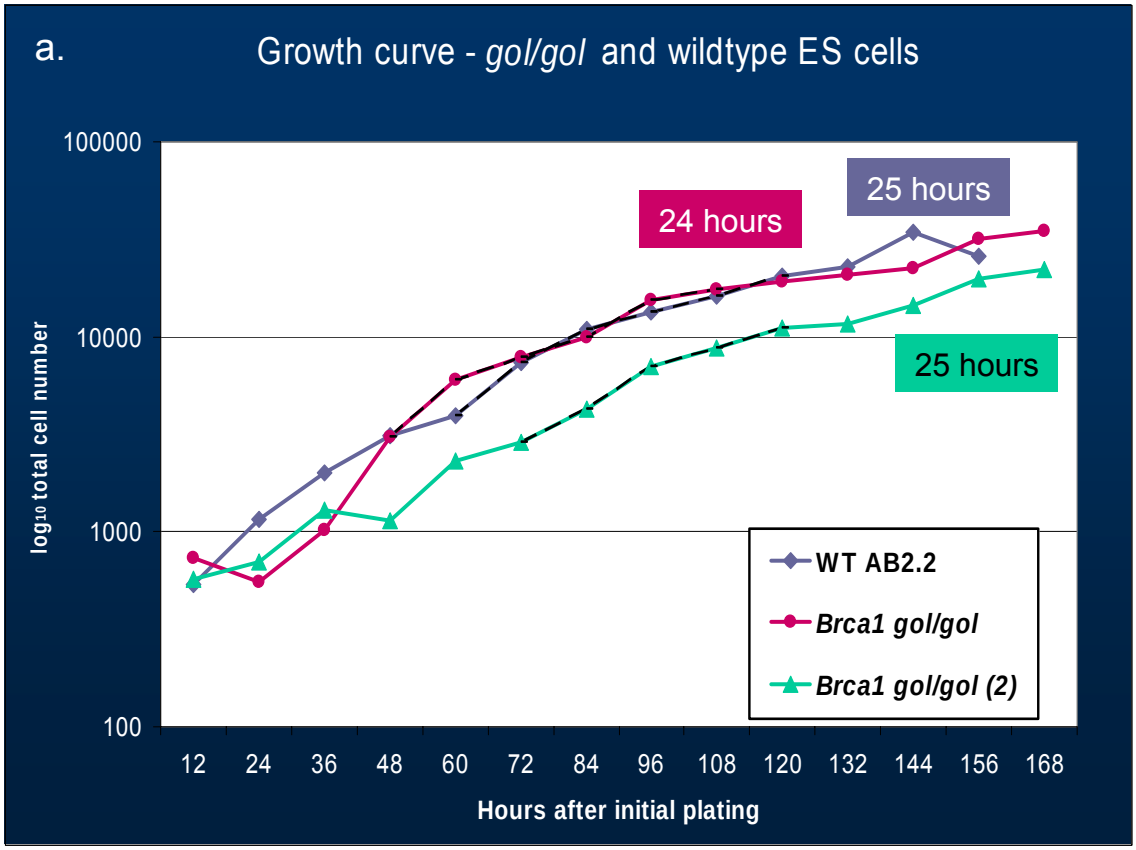
5.2 RESULTS

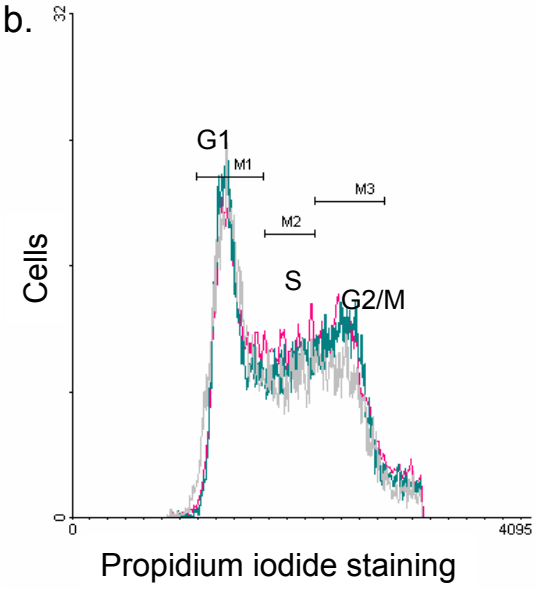
5.2.1 *gol/gol* ES cells grow normally compared to wildtype ES cells

To ascertain if *gol/gol* ES cells have a growth defect, the doubling times of *gol/gol* and wildtype ES cells in culture were established. Duplicate samples

of each cell line were counted every twelve hours for seven days. Figure 5.1 shows the resulting growth curves. Doubling times were calculated using the formula $DT=(t_1-t_0)/3.3 \log_{10}(N_1/N_0)$, where t_0 and N_0 represent the first time point and the number of cells at the first time point, respectively, and t_1 and N_1 represent a second time point and the number of cells at that time, respectively. Doubling times calculated for the period of log-phase growth (a straight line on a semi-log plot) were: 25 hours (wildtype), 24 hours (*gol/gol*) and 25 hours (*gol/gol 2*). Doubling times calculated over the entire seven days were: 26 hours (wildtype), 28 hours (*gol/gol*), and 30 hours (*gol/gol 2*). In either case, these are not appreciably different. This assay was only performed once. The same two *gol/gol* ES cell lines were used in the rest of the experiments described in this chapter.

A growth curve alone does not address potential changes in cell-cycle timing or the possibility of a block or delay in one part of the cell cycle. This has a bearing on the DNA damage assays described in this chapter, as the repair mechanism of DSBs depends in part on the phase of the cell cycle (see section 1.11.3.1.1). If *gol/gol* cells have a delay in going into S phase, DSBs may be more likely to be repaired by NHEJ, meaning that repair overall may be more error prone (and more detrimental), or that later assays used to determine the efficiency of HR and NHEJ in mutant cells would need to be interpreted with this bias in mind. Alternatively, if mutant cells have a shorter doubling time coupled with an increased amount of cell death, their growth curve might resemble that of wildtype cells. To partially allay these concerns, the cell-cycle profile of wildtype and *gol/gol* ES cells was determined by harvesting cells while in log-phase growth, labeling them with propidium iodide to measure DNA content, and performing flow cytometric analysis (see section 2.2.5.1 for details; flow sorting was performed by Bee Ling Ng of the Sanger Institute). Figure 5.1b shows overlapped cell-cycle profiles from wildtype and the two *gol/gol* ES cell lines. Markers show the regions used to calculate the percentage of cells in each phase; in all cases, the proportions were not different (values shown are an average of three experiments).





c.

Percentage of cells in each phase

	G1	S	G2/M
Wildtype (pink)	38 (1.25)	25 (0.94)	34 (1.63)
gol/gol (green)	42 (2.45)	25 (0.82)	31 (2.05)
gol/gol (2) (grey)	44 (2.08)	25 (1.00)	29 (2.08)

Figure 5.1: Growth curves and cell sorting of *gol/gol* and wildtype ES cells. **a.** Doubling times, as calculated from the log-phase period of growth (marked by dashed black lines on the curves), are indicated on the graph (see text for doubling time formula). Overall doubling times, calculated over the entire seven days were: 26 hours (wildtype), 28 hours (*gol/gol*), and 30 hours (*gol/gol(2)*). This assay was only performed once. **b.** Overlain cell-cycle profiles of wildtype (pink), *gol/gol* (green), and *gol/gol(2)* (grey) cells harvested in log-phase growth and stained with propidium iodide for DNA content. 20,000 cells were counted per cell line. A representative overlay is shown. **c.** Sorting experiments were done in triplicate and the averaged percentage of cells in various phases is shown (standard deviation in parentheses).

This experiment indicates that *gol/gol* ES cells do not appear to accumulate in one phase of the cell cycle, but it does not show whether cells with a S phase amount of DNA are actively synthesizing DNA. This was not determined, but could be done by pulse-labeling the cells with the thymidine analogue BrdU prior to harvest. As BRCA1 is known to have roles in checkpoint control, determination of the cell-cycle kinetics of the *gol/gol* cell lines may provide useful data about the mechanism of the defect in these cells.

5.2.2 *gol/gol* ES cells are not hypersensitive to UV treatment or H_2O_2-induced oxidative stress

Treatment of *gol/gol* ES cells with UV irradiation or H_2O_2 at a range of doses indicated that their colony-forming efficiency following treatment with these mutagens does not differ from that of wildtype cells. Figures 5.2 (UV) and 5.3 (H_2O_2) show the effect of these mutagens on the colony-forming ability of wildtype or *gol/gol* ES cell lines, normalized against the colony-forming ability of non-treated controls (plating efficiency at the “0” dose was thus set at “1”). All assays were performed at least in triplicate, with the error bars representing one standard deviation of the mean.

5.2.3 *gol/gol* ES cells are hypersensitive to γ-irradiation and mitomycin C (MMC) treatment

Exposure of *gol/gol* ES cells to γ-irradiation or the cross-linking agent MMC revealed that *gol/gol* ES cells are hypersensitive to both of these mutagens when compared to either wildtype or heterozygous ES cells. Figures 5.4 (γ-irradiation) and 5.5 (MMC) show the effects of these mutagens on the colony-forming ability of ES cells, normalized against that of non-treated controls. All assays were performed at least in triplicate, with the error bars representing one standard deviation of the mean.

At lower doses of γ-irradiation or MMC (100 or 250 rads or up to 1 μM MMC), *gol/gol* and wildtype ES cells have similar colony-forming abilities. For both mutagens, this similarity ends around the doses which kill ~50% of the

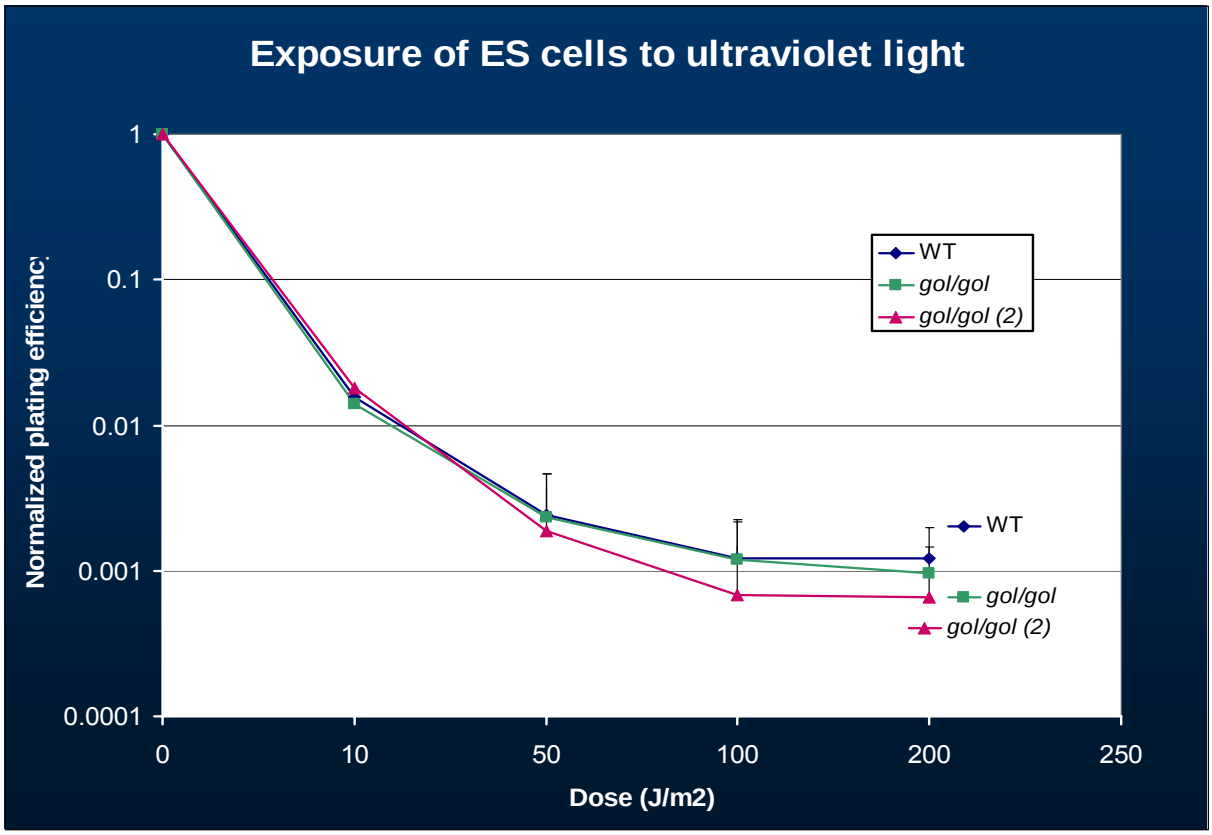


Figure 5.2: Plating efficiency of ES cells following UV exposure. *gol/gol*, *+/gol*, or wildtype ES cells were exposed to 0, 10, 50, 100, or 200 J/m^2 UVC. (254 nm at a power level of 40 watts/m^2). Plating efficiencies were normalized against the plating efficiency of non-treated controls. All assays were performed at least in triplicate. Error bars represent one standard deviation of the mean, but are only shown in the upward direction to simplify the graph.

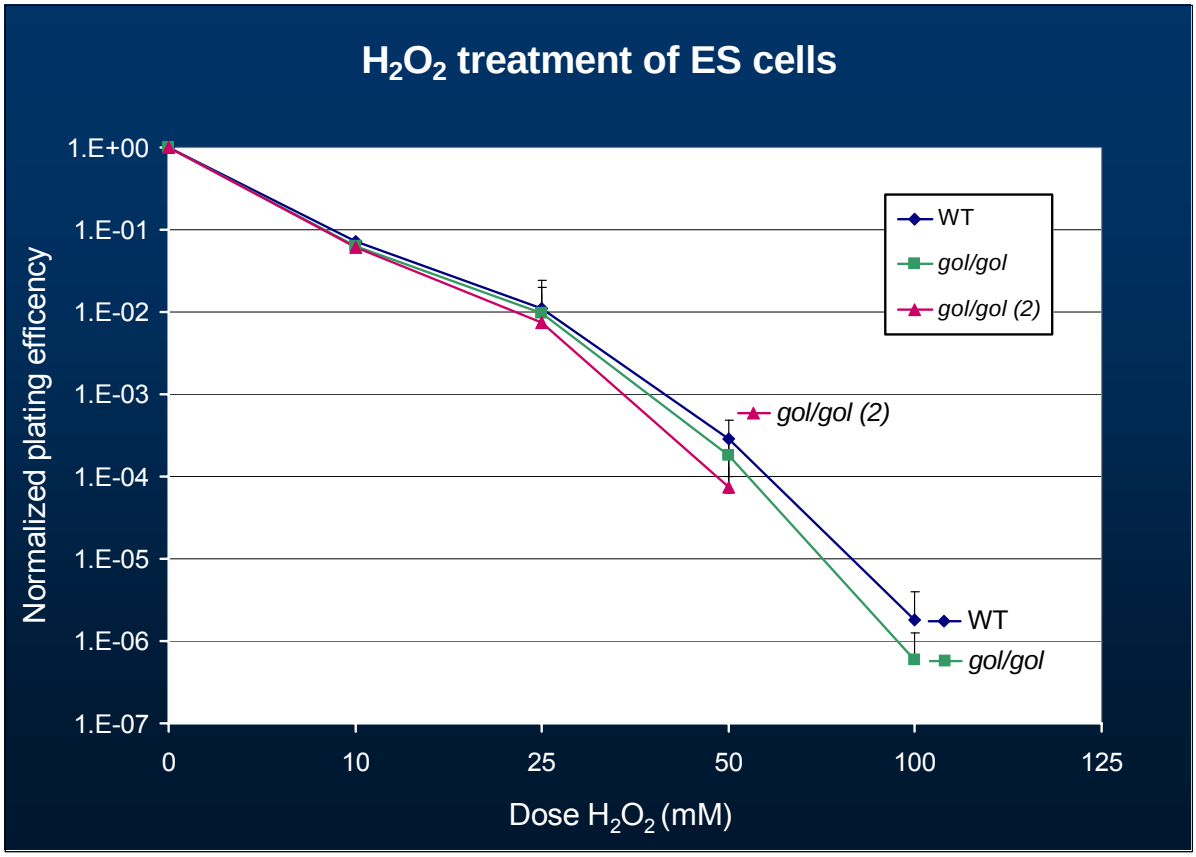


Figure 5.3: Plating efficiency of ES cells following H_2O_2 exposure. *gol/gol*, *+/gol*, or wildtype ES cells were exposed to 0, 10, 25, 50, or 100 mM H_2O_2 for 15 minutes. Plating efficiencies were normalized against the plating efficiency of non-treated controls. All assays were performed at least in triplicate. Error bars represent one standard deviation of the mean, but are only shown in the upward direction to simplify the graph.

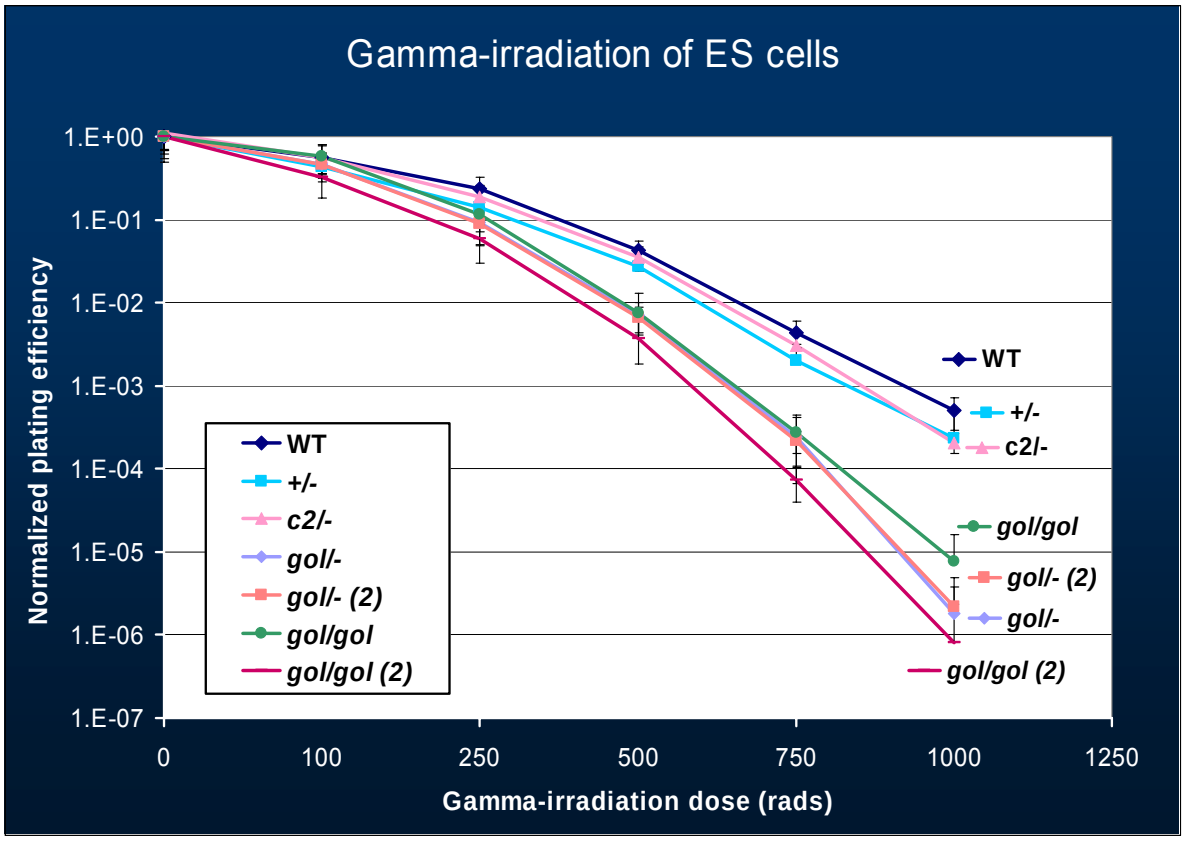


Figure 5.4: Plating efficiency of ES cells following γ-irradiation.
ES cells of the indicated genotypes were exposed to 0,100, 250, 500, 750, or 1000 rads of γ-irradiation (dose rate: 789 rads/minute). Plating efficiencies were normalized against the plating efficiency of non-treated controls. All assays were performed at least in triplicate. Error bars represent one standard deviation of the mean.

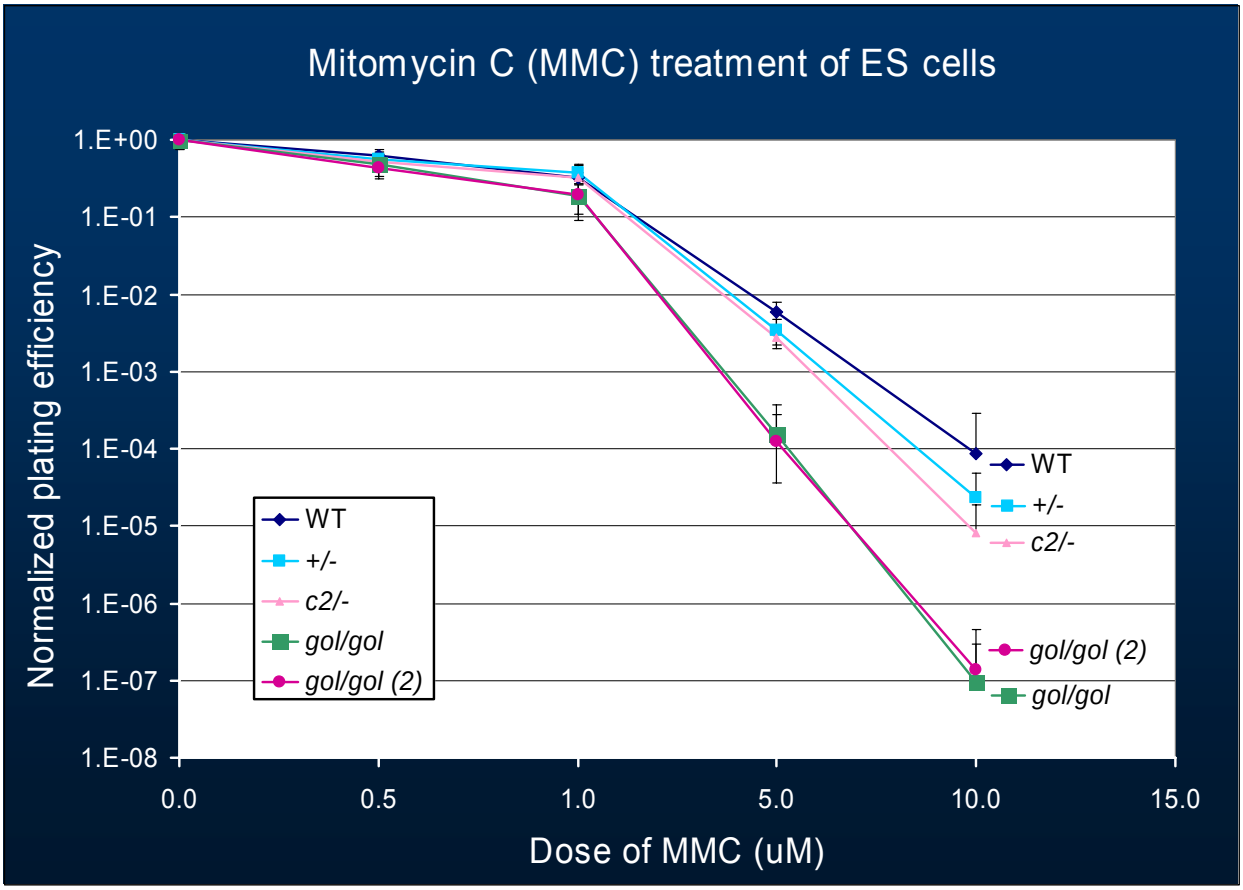


Figure 5.5: Plating efficiency of ES cells following MMC treatment. ES cells of the indicated genotype were exposed to 0, 0.5, 1, 5, or 10 μM MMC for four hours. Plating efficiencies were normalized against the plating efficiency of non-treated controls. All assays were performed at least in triplicate. Error bars represent one standard deviation of the mean.

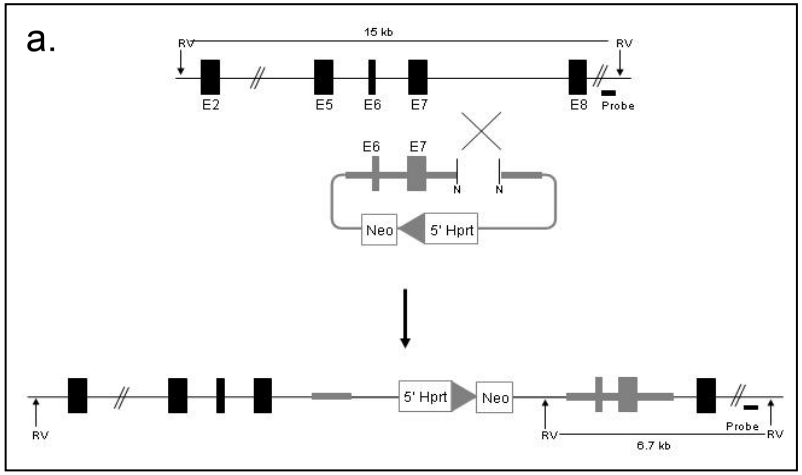
wildtype cells (100-250 rads, or 0.5–1 μM MMC). At higher doses, there is an increasingly large difference between the survival of *gol/gol* or *–/gol* and control cell lines. Colonies formed from *gol/gol* or *–/gol* cells given higher doses of MMC or γ-irradiation were consistently smaller than those formed by similarly-treated wildtype cells, and were routinely cultured for two extra days to allow the colonies to be scored more easily.

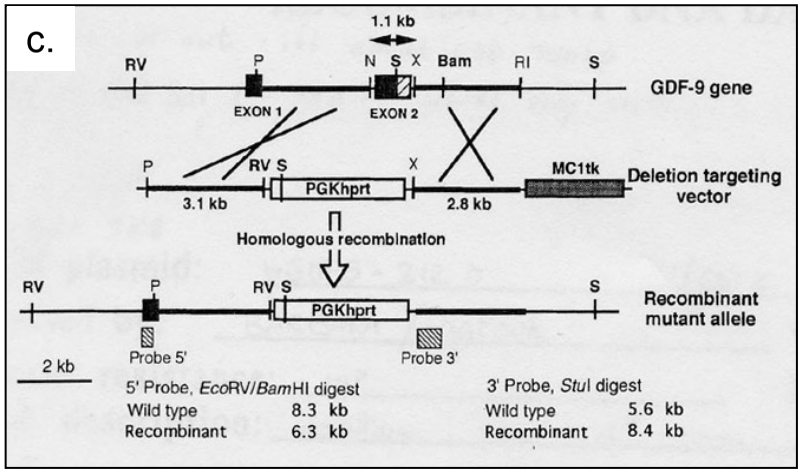
As discussed in section 1.11.3, the characteristic lesions formed after γ-irradiation or MMC treatment are repaired by DSBR mechanisms. The two major forms of DSBR are HRR and NHEJ (see Figure 1.13). The DSBR defect observed in *gol/gol* ES cells prompted further experiments to assess the efficiency of homologous recombination and NHEJ in these cells.

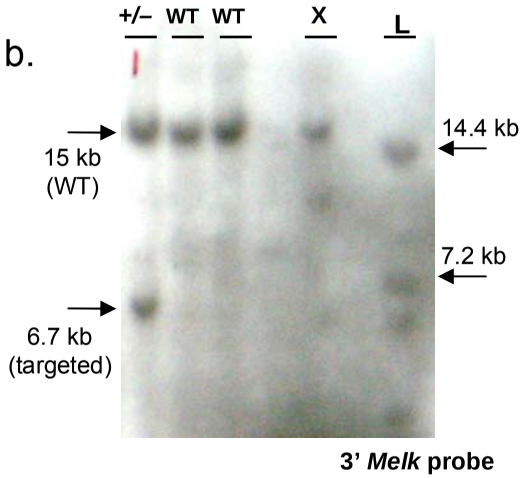
5.2.4 *gol/gol* ES cells have a small deficiency in gene-targeting efficiency

Homologous recombination repair is commonly tested one of several methods, including by I-SceI assay (I-SceI assays add a recognition site for the rare-cutting I-SceI endonuclease to the cell, either by direct integration into the genome or on a plasmid; transfection of the nuclease into cells generates the break. Generally, accurate repair of the break is measured by reconstitution of a selection cassette), pulsed-field gel electrophoresis, or by measuring the efficiency of gene targeting. A number of groups within the BRCA field have used gene targeting to look at HRR (Essers, 1997; Rijkers, 1998; Jasin, 2002). In studies using this and other assays, the results of the two, while differing in fold-induction, generally show the same trend.

The efficiency of homologous recombination in *gol/gol* and wildtype ES cells was tested by gene targeting using both a replacement vector (for *Gdf-9*, a gift from Marty Matzuk of Baylor College of Medicine, Houston, TX (Dong, 1996)), and an insertion vector (for *Melk*, isolated from the 5' *Hprt* targeting vector library (Zheng, 1999a) by a colleague, Jyh-Yih Chen). Screening for correctly-targeted colonies was done by mini-Southern (Figure 5.6). Figure







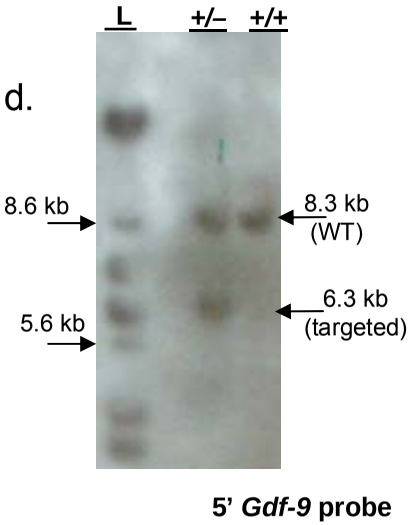


Figure 5.6: Targeting *Gdf-9* and *Melk*. **a.** The *Melk* targeting vector and probe schematic, modified from a diagram provided by Dr. Louse van der Weyden. **b.** Representative mini-Southern blot showing targeted and non-targeted *Melk* clones. **c.** The *Gdf-9* targeting vector and probe schematic, taken from (Dong, 1996). **d.** Representative mini-Southern blot showing targeting and non-targeted *Melk* clones. L=ladder, WT=wildtype, RV=*Eco*RV, N=*Nde*I, X=incorrect targeting.

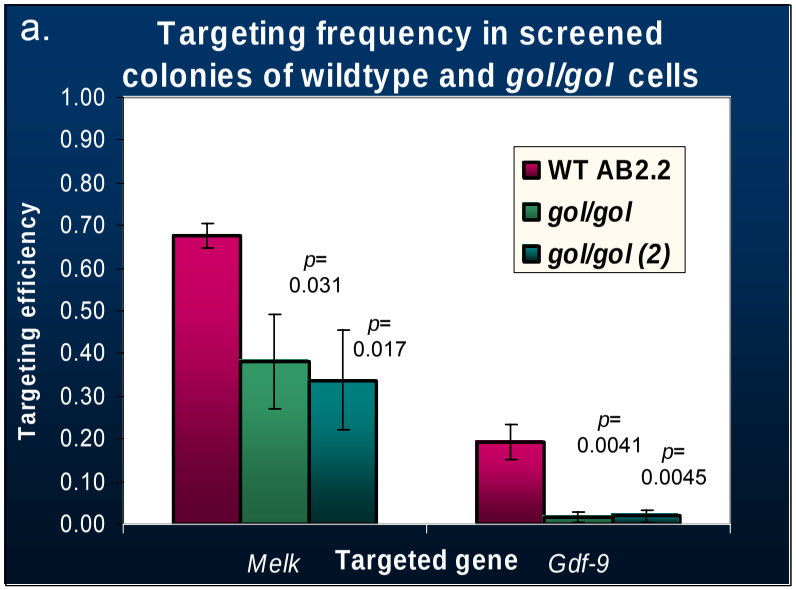
5.7a shows that the targeting frequency of *gol/gol* ES cells is lower than that of wildtype cells for both genes. The average of three experiments is plotted; error bars represent one standard deviation from the mean. A two-tailed t-test indicates that the difference between the targeting efficiency of either *gol/gol* ES cell line and the wildtype control is statistically significant (a *p*-value of less than 0.05 is considered significant). However, once these values are corrected for random integration (as measured by the total number of drug-resistant colonies resulting from an entire *Melk* electroporation, Figure 5.7b), the absolute targeting frequencies of *gol/gol* and wildtype cells are more similar (Figure 5.7c), and although they are still lower than that of wildtype cells, the difference is smaller, ranging from 1.2-fold to 5.6-fold lower.

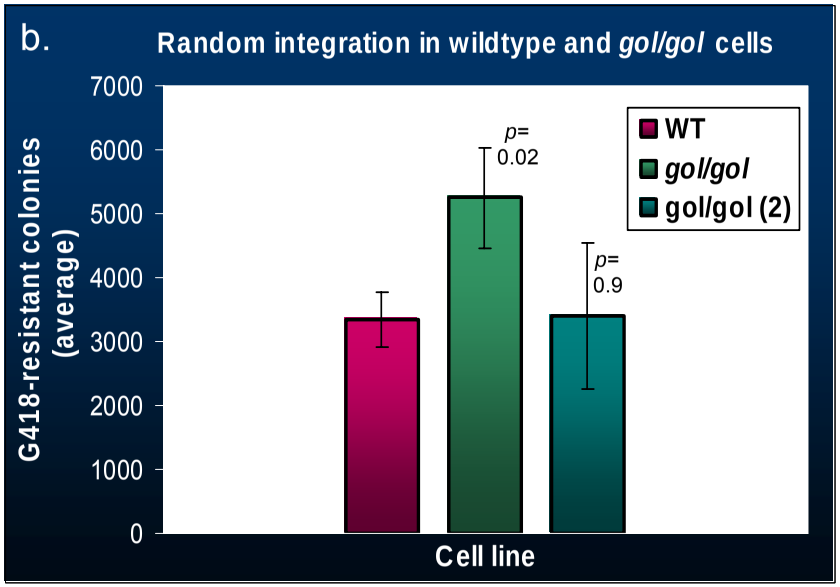
5.2.5 *gol/gol* ES cells have an increase in NHEJ efficiency

Assaying NHEJ is commonly done by one of several methods – I-SceI rejoining, direct integration of plasmids, plasmid-based transfection assays (in cells or using cell-free extracts), pulsed-field gel electrophoresis-based measurement of repair kinetics, or by measuring the response to retroviral insertion (Daniel, 1999; Moynahan, 1999; Li, 2001; Willers, 2002; Zhong, 2002a; Zhong, 2002b).

The NHEJ efficiency of *gol/gol* ES cells was tested using the gene trap vector pGT designed by Dr. William Skarnes (Wellcome Trust Sanger Institute, Hinxton, UK). The trap cassette consists of a promoterless *β-geo* gene (a fusion of *Neo* and *β-gal*) proceeded by a splice acceptor from the mouse *engrailed-2* gene (Figure 1.17). *β-geo* lacks an ATG start site, and must be successfully spliced into a transcript before it can be expressed. pGT is randomly integrated into the genome by direct electroporation into ES cells, meaning that this assay for random integration does not depend on any intermediate steps, as would be the case in retroviral-mediated delivery of a gene trap cassette (Skarnes, 2000).

Both of the *gol/gol* ES cell lines have an increased efficiency of NHEJ as measured by gene trapping (Figure 5.8). A two-tailed t-test indicates that this





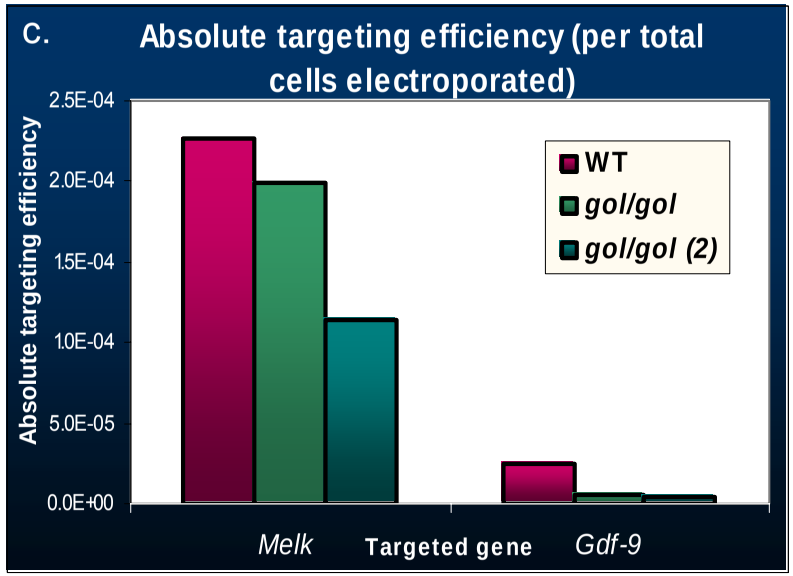


Figure 5.7: Targeting efficiencies of wildtype and *gol/gol* ES cells. **a.** The average targeting efficiency of three targeting experiments per gene per cell line is graphed. **b.** Random integration of the *Melk* targeting vector in WT and *gol/gol* ES cells. The average number of drug-resistant colonies from three experiments is graphed. **c.** The absolute targeting efficiency of each cell line (per 10^7 cells electroporated), taking both the targeting frequency and the amount of random integration into account.
Error bars represent one standard deviation of the mean.
p-values were calculated using a two-tailed t-test and compare the indicated *gol/gol* sample to the wildtype control.
WT= wildtype.

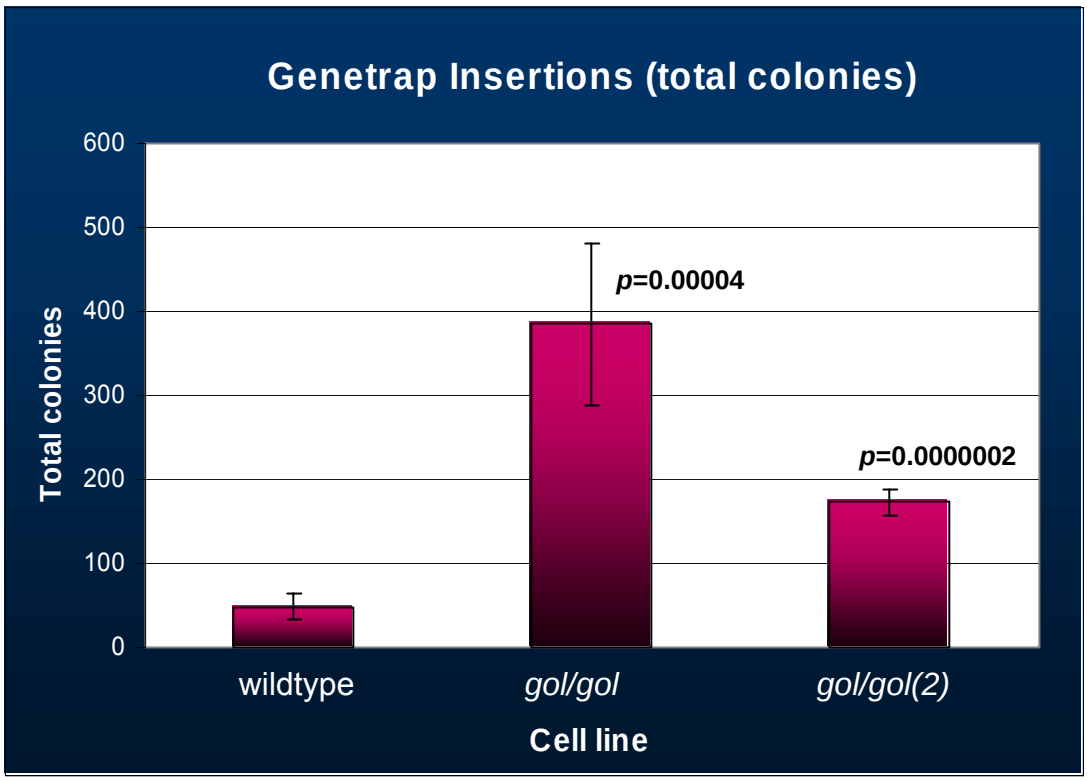


Figure 5.8: NHEJ efficiencies of wildtype and *gol/gol* ES cells.
The average total colony number from three experiments per cell line is graphed. Error bars indicate the standard deviation. Statistical significance of the result from each *gol/gol* cell line compared to the wildtype control was measured by a two-tailed t-test. *p*-values are shown (a *p*-value less than 0.05 is considered significant).

increase is statistically significant. The average of three experiments is plotted; error bars represent one standard deviation from the mean.

5.2.6 Brca1^{gol} cellular localization

In order to determine if Brca1^{gol} is found in the nucleus, immunolocalization studies were performed using *gol/gol* and wildtype cells (immunolocalization and confocal microscopy were performed by Michal Goldberg of the Wellcome Trust/Cancer Research UK, Cambridge). As ES cells are very small and a *gol/gol* mouse model did not yet exist from which MEFs could be generated, *gol/gol* and wildtype ES cells were differentiated in culture as described previously (Robertson, 1987) to provide larger cells. The antibody used in these studies was obtained from a commercial vendor (M-20, from Santa Cruz Biotechnology), raised to a peptide from the C-terminus of murine Brca1. Previous immunolocalization studies using this antibody have demonstrated a nuclear and cytoplasmic localization profile of Brca1, similar to results obtained with other antibodies (Bachelier, 2000). The specificity of this antibody has been at least partially demonstrated in a study designed to test the specificity of a new BRCA1 antibody: the new antibody was used in an immunoprecipitation experiment, then the immunoprecipitates were subjected to Western blotting and probed with a panel of commercially available BRCA1 antibodies. M-20 performed identically to the other BRCA1 antibodies, including the widely-used Ab-1 antibody, but Western blot evidence shows that all the antibodies recognize not only the wildtype and ΔX.11 forms of Brca1 but at least two other bands which may be non-specific or may arise from alternative splicing of the *Brca1* gene (Zhang, 1997).

As indicated in Figure 5.9, Brca1 is located in both the nucleus and the cytoplasm in both wildtype (5.9a) and *gol/gol* (5.9b) cells. In addition, Brca1 nuclear foci are observed in undamaged cells of both genotypes (Figure 5.10), in accordance with what has been observed in numerous other BRCA1 immunolocalization studies (*e.g.* (Scully, 1997c; Wang, 2000b; Huber, 2001)). The amount of focus formation may be lower than it appears, as the overall punctate appearance of the immunolocalization pattern may lead to an

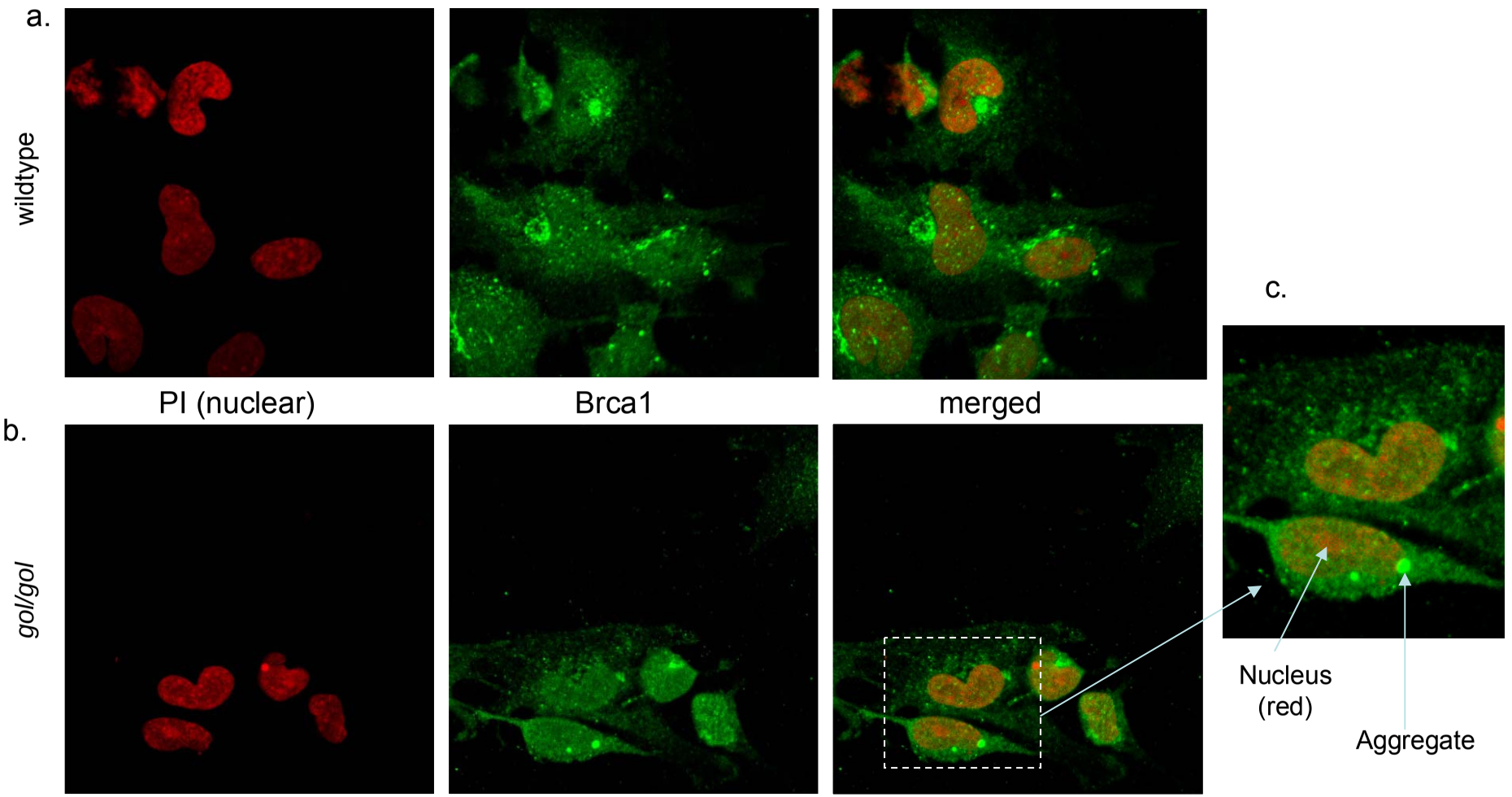


Figure 5.9: Nuclear localization of Brca1 in *gol/gol* and wildtype cells.
a. Wildtype cells. **b.** *gol/gol* cells. **c.** Zoom-in on one *gol/gol* cell to show an aggregate in an untreated cell. Brca1 staining is shown in green, nuclear staining with propidium iodide (PI) in red. Immunlocalization and confocal microscopy were performed by Michal Goldberg (see Methods). The Brca1 antibody M-20 (Santa Cruz) was used.

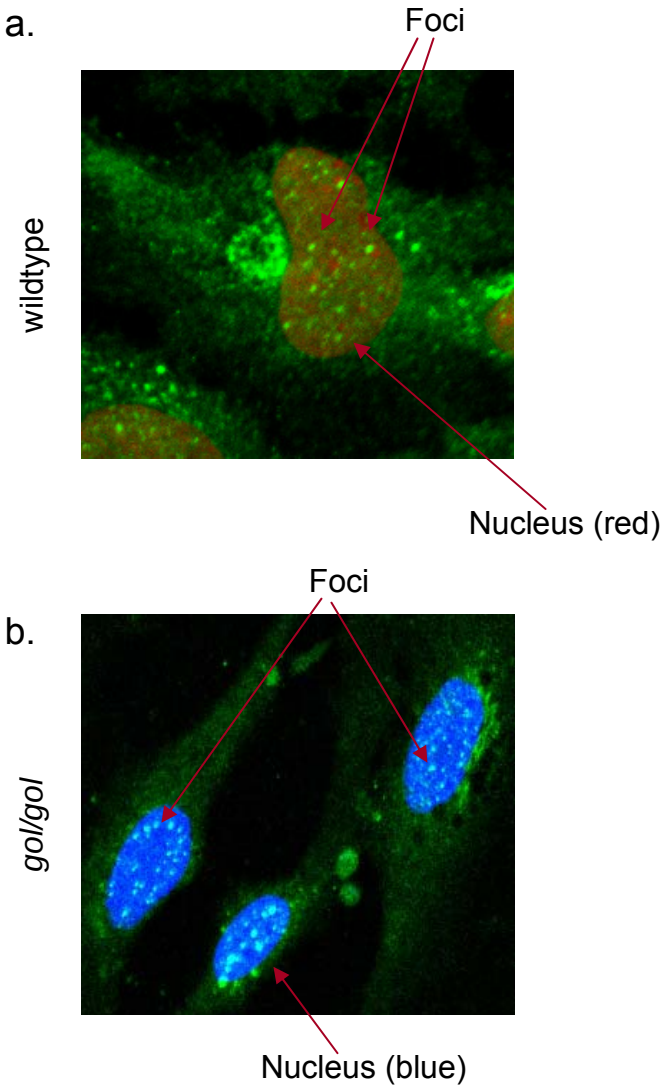


Figure 5.10: Nuclear foci in undamaged cells.
a. Wildtype cells. Brca1 staining is shown in green, nuclear staining with propidium iodide (PI) in red.
b. *gol/gol* cells. Brca1 staining is shown in green, nuclear staining with TOTO-3 iodide in blue. Both nuclear and cytoplasmic localization of Brca1 is observed, and nuclear foci are observed in cells of both genotypes. Immunlocalization and confocal microscopy were performed by Michal Goldberg (see Methods). The Brca1 antibody M-20 (Santa Cruz) was used.

overestimation of focus formation. Some *gol/gol* cells additionally have larger aggregates of protein near the nucleus or in the cytoplasm, but such aggregates were also seen in wildtype cells (inset Figure 5.9 and Table 5.1). Oddly-shaped nuclei are a normal characteristic of differentiated cells and are seen in both the *gol/gol* and wildtype cell lines. It is possible that levels of Brca1 protein may be higher in *gol/gol* cells, although no attempt at formal quantification was made. The level of cytoplasmic Brca1 localization, although consistent between wildtype and *gol/gol* cell lines, is higher than has been observed in other studies, and overall localization is fairly punctate. These observations are not constant with previous immunolocalization studies, and the presence of aggregates has not been reported previously. These discrepancies may result from the use of differentiated cells, which have not been used in previous studies, or from the antibody used. As discussed above, the antibody may recognize non-specific polypeptides, and before this experiment can be interpreted further, either use of another antibody, or a repeat of the experiment but using competitor Brca1 peptide to assess the specificity of the antibody should be done.

5.2.7 Localization patterns of Brca1^{gol} and wildtype Brca1 differ after γ-irradiation but are similar following UV treatment

The localization of Brca1 in nuclear foci following various forms of DNA damage is well-documented (Scully, 1996; Scully, 1997b). In this study both normal and damage-induced Brca1 nuclear foci were observed. Following exposure to UV irradiation, such foci were seen in a similar percentage of *gol/gol* and wildtype cells (Figure 5.11/Table 5.1). As mentioned previously, though the overall punctate appearance of the immunolocalization pattern may lead to an overestimation of the number of foci formed.

Following γ-irradiation, both *gol/gol* and wildtype cells also exhibit nuclear foci (Figure 5.12). However, ~25% of *gol/gol* cells have large, generally perinuclear, aggregates of protein (inset, Figure 5.12 and Table 5.1). These aggregates are rarely observed in UV-treated cells of either genotype, or in γ-irradiated wildtype cells. About 10% of untreated wildtype and 16% of

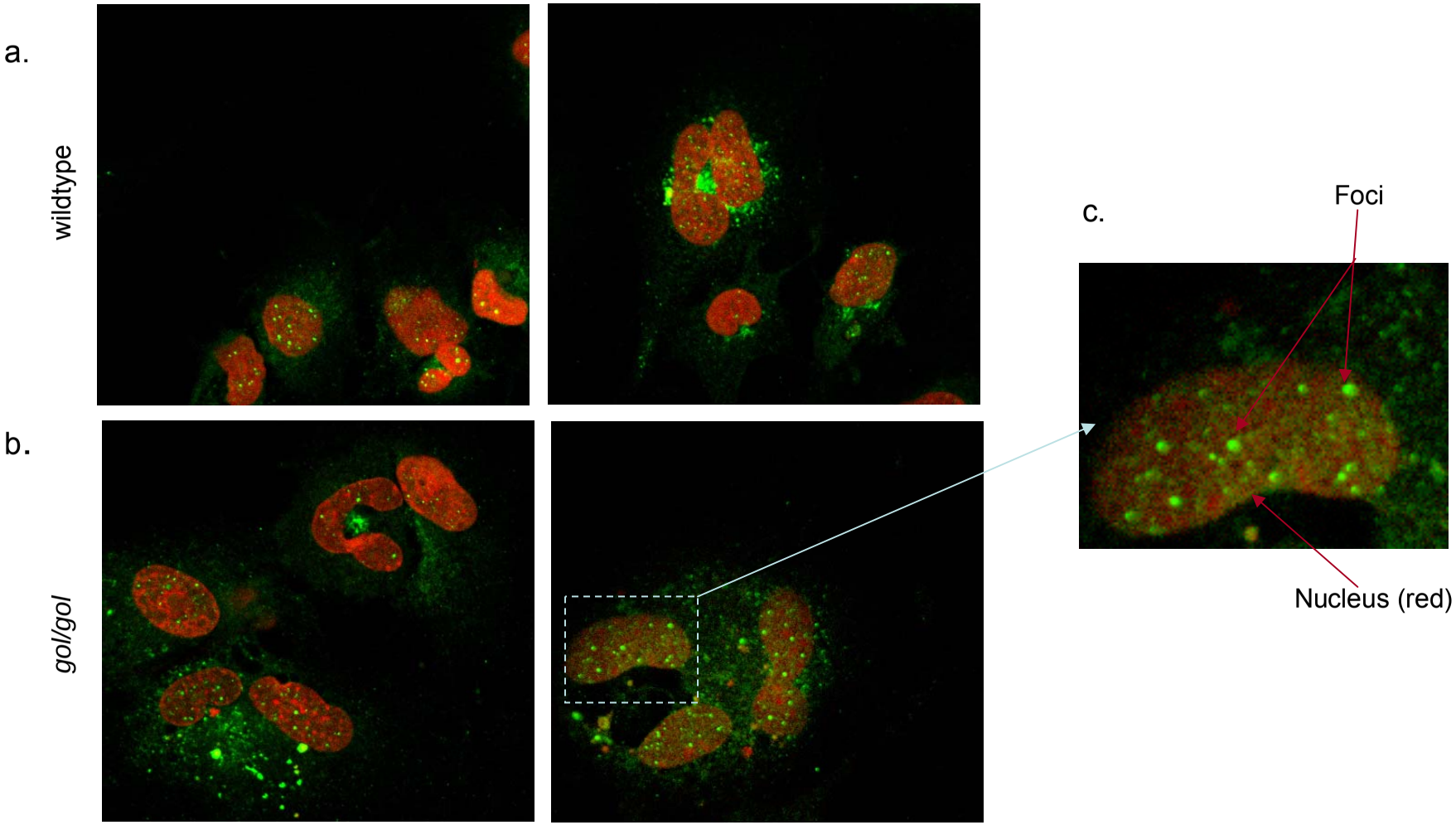


Figure 5.11: Nuclear localization of Brca1 in *gol/gol* and wildtype cells following UV exposure.
a. Wildtype cells. **b.** *gol/gol* cells**. c.** Zoom-in on a single *gol/gol* cell to show nuclear foci. Brca1 staining is shown in green, nuclear staining with propidium iodide (PI) in red; all images are shown as merges of both stains. Immunlocalization and confocal microscopy were performed by Michal Goldberg (see Methods). The Brca1 antibody M-20 (Santa Cruz) was used.

Table 5.1: Foci and aggregate formation in *gol/gol* or wildtype cells.
Foci and aggregates in *gol/gol* or wildtype cells, both untreated or following exposure to ultraviolet (UV)- or γ-irradiation. Nuclei were scored positive for foci if six or more foci were observed, and positive for aggregates if one large or several smaller aggregates were present.

Cell line	Treatment	Nuclei	Foci	Percentage of cells with foci	Aggregates	Percentage of cells with aggregates
Wildtype	none	117	46	39	12	10
gol/gol	none	89	18	20	14	16
Wildtype	γ-irradiation	102	48	47	2	2
gol/gol	γ-irradiation	51	31	61	13	25
Wildtype	UV	37	29	78	1	3
gol/gol	UV	35	24	69	1	3

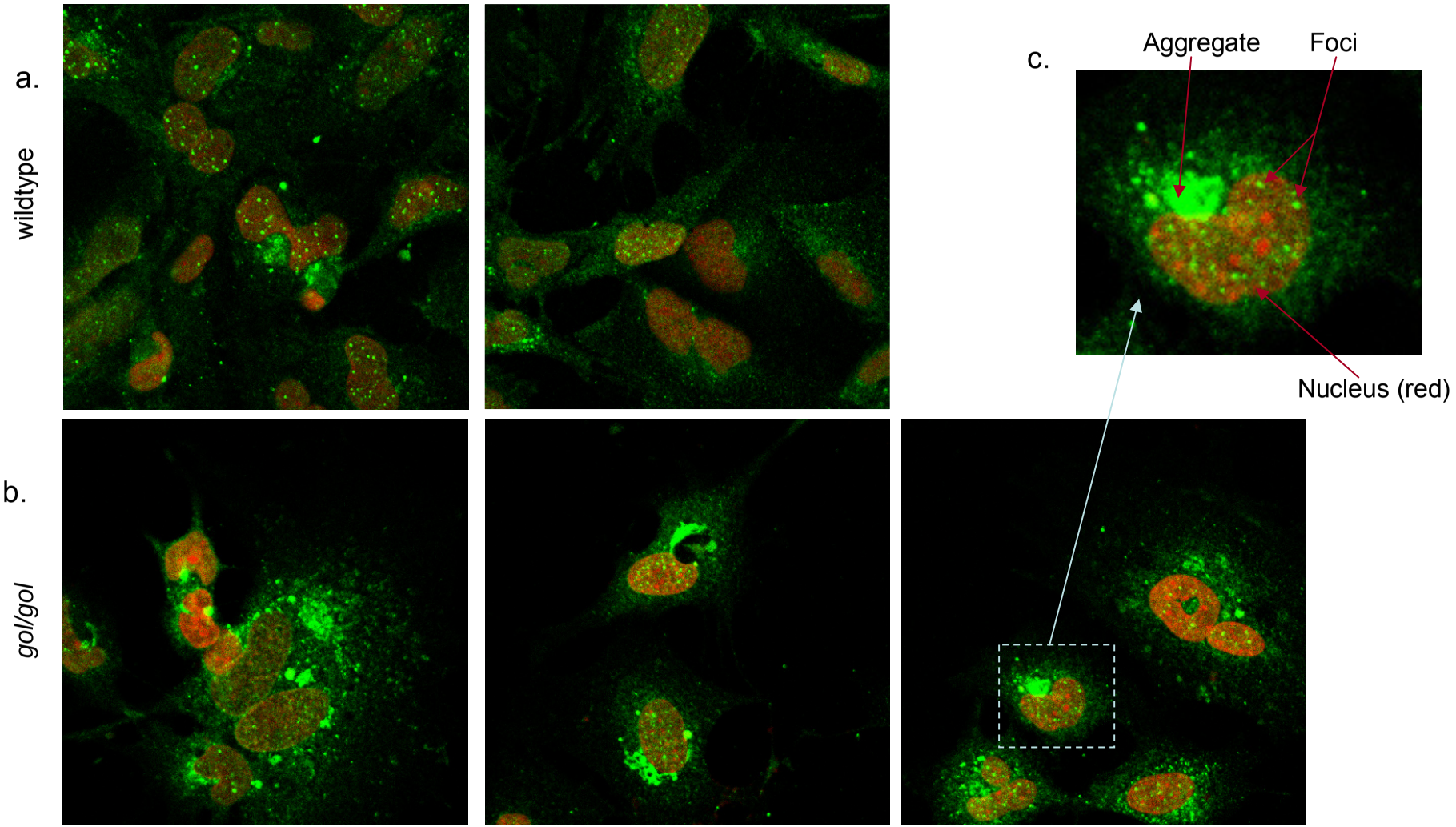


Figure 5.12: Nuclear localization of Brca1 in *gol/gol* and wildtype cells following γ-irradiation.
a. Wildtype cells. **b.** *gol/gol* cells. **c.** Zoom-in on one *gol/gol* cell to show nuclear foci and an aggregate. Brca1 staining is shown in green, nuclear staining with propidium iodide (PI) in red; all images are shown as merges of both stains. Immunlocalization and confocal microscopy were performed by Michal Goldberg (see Methods). The Brca1 antibody M-20 (Santa Cruz) was used.

untreated *gol/gol* cells exhibit aggregates, but they are generally smaller than the aggregates seen in γ-irradiated *gol/gol* cells (inset Figure 5.9). The reason for aggregate formation, or whether they have any functional consequences, is unknown. It is possible that they are a consequence of overexpression of the Brca1^{gol} protein, or may indicate that Brca1^{gol} is more stable than wildtype Brca1 or degraded inefficiently. As only one antibody was used in these immunolocalization studies, it is additionally possible that the aggregates are an artifact of this antibody, although such aggregates were not reported by another group using the same antibody (Bachelier, 2000).

5.3 DISCUSSION

5.3.1 Brca1^{gol} and base repair

This chapter describes a series of experiments designed to investigate the response of *gol/gol* ES cells to various forms of DNA damage. It was first shown that *gol/gol* cells have a colony-forming ability comparable to that of wildtype cells following UV irradiation or oxidative stress from exposure to H_2O_2.

There are several indications that BRCA1 is involved in the response to base damage. One is the reaction of the protein to these types of damage – BRCA1 appears to be modified by phosphorylation and dispersed from normal S phase foci following UV or H_2O_2 exposure (Scully, 1997b; Thomas, 1997; Okada and Ouchi, 2003). Experimental evidence indicates a role for BRCA1 in transcription-coupled repair (TCR) of oxidative damage. 8-oxo-guanine lesions are generally repaired by base excision repair (BER), but repair of the mutation in actively transcribed genes occurs through a TCR pathway (Le Page, 2000a). Human HCC1937 cells, which lack both wildtype BRCA1 and p53, appear to be deficient in TCR of an 8-oxo-guanine lesion transfected into the cells on a plasmid. Adenoviral-based overexpression of *BRCA1* rescues this TCR defect (Le Page, 2000b). *Brca1*$^{\Delta X.11/\Delta X.11}$ ES cells (allele described in Table 1.3 #5) appear to be hypersensitive to oxidative damage from H_2O_2 exposure, and *Brca1*$^{\Delta X.11/\Delta X.11}$*, p53*$^{-/-}$ MEFs are also

hypersensitive to H_2O_2 exposure compared to *Brca1*$^{+/\Delta X.11}$, *p53*$^{-/-}$ MEFs. Some evidence indicates that this may result from a TCR deficiency (Gowen, 1998; Cressman, 1999a; Gowen, 2003).

These *Brca1*$^{-/-}$, *p53*$^{-/-}$ MEFs may also be more sensitive to UV exposure when compared to *Brca1*$^{+/-}$, *p53*$^{-/-}$ MEFs (the difference was not statistically significant at all doses tested) (Cressman, 1999a). The involvement of BRCA1 in global genomic repair (GGR) of UV-induced lesions has been suggested by an experiment involving tetracycline-controlled overexpression of *BRCA1* in *p53*$^{+/+}$ or *p53*$^{-/-}$ human cell lines (Harkin, 1999). In *p53*$^{-/-}$ cells, UV-induced lesions are repaired efficiently by TCR, but cannot be repaired by GGR. When *BRCA1* was overexpressed in *p53*$^{-/-}$ cells, UV-induced lesions on the non-transcribed strand were repaired efficiently. Overexpression of *BRCA1* in *p53*$^{+/+}$ cells did not significantly change the amount of repair on either strand (Hartman and Ford, 2002).

Data generated using the *gol/gol* ES cells contrasts with the studies described above, which may indicate either that the N-terminal region mutated in Brca1^{gol} is not required for the response of cells to base damage (i.e., triggering cell-cycle checkpoints or aiding in the actual repair) or that the *gol* mutation allows the cell to ignore or tolerate mutated bases. However, if it is the case that the *gol/gol* cells are able to ignore base damage, it might be expected that these cells would appear to be hyposensitive to damage at higher doses compared to wildtype cells. This was not observed. The lack of hypersensitivity to base damaging agents may be somewhat surprising, as damaged bases are capable of generating double-strand breaks in following replication errors or polymerase stalling. For this reason, it would be worthwhile to determine the cell-cycle kinetics of the *gol/gol* cells both before and after DNA damage to determine if they undergo cell-cycle arrest as would normally occur. If undamaged mutant cells normally have a delay in S phase progression, then UV-irradiated mutant cells may appear to have a normal repair response simply because the delay allows additional time for repair. Figure 5.1b suggests that such a delay is not taking place, but BrdU labeling

of the cells to confirm that S phase is occurring normally should be done to support these data further.

It should also be noted that, in contrast to previous studies of the involvement of Brca1 in the response to base damage, *gol/gol* cells do not have known secondary mutations. This may be a relevant point, because *p53*-deficient cell lines are known to have a deficiency in GGR of UV-induced damage (Hartman and Ford, 2002), and p53 upregulates several genes (including *p21*) following UV exposure (el-Deiry, 1993). While *p53*-deficient cells are only slightly hypersensitive to H_2O_2 treatment (Yin, 1998; Lin, 2000), p53 is stabilized and upregulates *p21* in response to H_2O_2 treatment (Chen, 2003). BRCA1 can also upregulate *p21* in response to DNA damage, in a p53-independent manner, and overexpression of *BRCA1* has been shown to upregulate *p21* expression (Somasundaram, 1997; MacLachlan, 2000b). It is therefore possible that the hypersensitivity of cells lacking p53 and BRCA1 (such as the HCC1937 cell line or the *Brca1*$^{-/-}$, *p53*$^{-/-}$ MEFs described above) to UV irradiation or H_2O_2 exposure is due at least in part to loss of p53, and that expression/overexpression of *BRCA1* rescues the phenotype by independently activating *p21*.

5.3.2 Brca1^{gol} and DSBR

gol/gol ES cells are hypersensitive to MMC and γ-irradiation, both of which cause lesions repaired by the DSBR pathway. These results are consistent with numerous other studies confirming the involvement of BRCA1 in DSBR (reviewed in Jasin, 2002; Thompson and Schild, 2002). The consistent phenotype of γ-irradiation hypersensitivity in *BRCA1*-mutant cells, regardless of the area mutated, has led to the suggestion that multiple domains of BRCA1 are necessary for the response to this mutagen (Scully, 1999). As BRCA1 has been linked to both HRR and NHEJ, the efficiency of both of these processes in *gol/gol* ES cells was queried.

5.3.2.1 gol/gol cells and HRR

Various assays have been used to assess HRR in cell lines, including I-SceI assays, PFGE-monitored repair kinetics following DNA damage, and gene-targeting in cells.

The I-SceI system has the advantage that it measures repair of an induced DSB, modeling a damage-induced break in the genome, and several groups have demonstrated that ability to repair an I-SceI break parallels the radiation-sensitivity of the cell line (Johnson and Jasin, 2001). However, it is acknowledged that the break generated may not be the most accurate model of a radiation-induced DSB: the I-SceI endonuclease generates a directly re-ligatable set of 4-bp overhangs which retain their 3' hydroxy groups; DSBs generated by γ-irradiation are likely to be more diverse and include blunted ends as well as ends lacking hydroxy groups (Willers, 2002). Pulsed-field gel electrophoresis (PFGE)-based monitoring of DBS repair following damage can be used to assess the overall amount of repair, and has the advantage that it can be used to measure the kinetics of repair of radiation-induced breaks.

Gene targeting can also be used as an assay for the efficiency of homologous recombination, but has the disadvantage of not measuring repair of an induced break in the genome. In the mouse, proving that HRR genes are involved in gene targeting is not completely straightforward, as many of the key genes involved generate embryonic lethal mouse knockouts (*Rad51, Rad50, Mre11*) (Lim and Hasty, 1996; Xiao and Weaver, 1997; Luo, 1999). However, *Rad54* knockout mice are viable. *Rad54*$^{-/-}$ ES cells are hypersensitive to DSB-inducing agents such as γ-irradiation and MMC, and gene targeting is markedly decreased in these cell lines (7-10 fold, using 2 targeting vectors) (Essers, 1997). *Rad52*-deficient mice are also viable. *Rad52*$^{-/-}$ ES cells are not hypersensitive to ionizing radiation, and have only a slight decrease in targeting efficiency (Rijkers, 1998). These data suggest that targeting efficiency may be linked to radiation sensitivity, and supports the use of gene targeting as an assay for HRR. However, it is possible that that

an overlapping yet distinct set of proteins mediate homology-directed repair and homology-directed integration of targeting vectors.

gol/gol ES cells have a slight decrease in homologous recombination efficiency as measured by gene targeting (Figure 5.7). A deficiency in homologous recombination is in agreement with several similar studies using *Brca1*$^{\Delta X.11/\Delta X.11}$ ES cells (allele described in Table 1.3 #5) (Moynahan, 1999; Moynahan, 2001). The ES cells used by Moynahan *et al.* appear to be more severely deficient in homologous recombination than are *gol/gol* ES cells; the former had almost undetectable gene-targeting efficiencies compared to wildtype or heterozygous cells, with a 13-fold difference in targeting efficiencies once random integration was factored in. *gol/gol* ES cells showed a more modest 1.2- to 5.6-fold decrease compared to wildtype cells (Moynahan, 1999; Moynahan, 2001). However, when Moynahan *et al.* used an I-SceI assay to measure the repair of DSBs by HRR, their mutant cells were 5- to 6-fold less efficient at repair than wildtype cells. The results of this assay correlate well with the results generated using *gol/gol* ES cells (Moynahan, 1999). However, there is a difference between the gene targeting efficiencies of the two *gol/gol* mutant cell lines used in the assays, something that promotes consideration of another assay for confirming the results.

5.3.2.2 gol/gol cells and NHEJ

NHEJ is commonly assessed by one of several methods – I-SceI rejoining, direct integration of plasmids, plasmid-based transfection assays (in cells or using cell-free extracts), PFGE-based measurement of repair kinetics, or by measuring the response to retroviral insertion (Daniel, 1999; Moynahan, 1999; Li, 2001; Willers, 2002; Zhong, 2002a; Zhong, 2002b). Although the I-SceI assay again has the advantage of being break-induced, this assay generally involves selection of a reconstituted selection cassette. Error-prone NHEJ generally will not reconstitute such a cassette, meaning that amplification/sequencing of the cassette (or some other method of physical determination) must be carried out to determine if rejoining has occurred. Additionally, accurate NHEJ may be classified as HRR if selection of a

reconstituted cassette is used (Willers, 2002). Plasmid substrates, used in cell-free or *in vitro* assays, also commonly need to be recovered for analysis, and these substrates may be subject to nuclease attack when introduced into cells.

Random integration of a linearized plasmid carrying a selection marker into the genome of cells has also been used to assess the efficiency of NHEJ. This assay has the disadvantage of not measuring the response to a damage-induced DSB. However, there are indications that integration of a plasmid into the genome utilizes the key proteins in NHEJ (although other factors are also likely to be involved). Insertion of the linearized vector is assumed to take place at a DSB in the genome. This supposition is supported by early studies which demonstrated that insertion of a plasmid does not take place at genomic sites carrying any homology to the insert. Instead, insertion sites showed microhomologies (1 or 6 bp) at the joining junction, or short deletions, both of which are consistent with the mechanism of NHEJ (Murnane, 1990; Lieber, 2003). Further, sequencing-based studies of repair at induced genomic breaks (I-SceI sites) showed that about 8% of repaired sites had "captured" extrachromosomal DNA – either part of the I-SceI expression plasmid itself, or, in a second study, ΦX174 fragments introduced into the cell with the I-SceI plasmid. Integration appeared to occur preferentially at the induced break-site and not elsewhere in the genome. Again, microhomologies of 1-4 base pairs were commonly seen at insertion sites, along with small deletions at the junctions (Lin and Waldman, 2001; Dellaire, 2002). Functional data generated using knockout cell lines also supports the idea that random integration of linear plasmids requires key NHEJ proteins: cell lines lacking DNA-PKcs (mouse scid lines), Ku80 (hamster xrs5), Nbs1 (mouse ES knockout cell line), or XRCC4 all have a lower efficiency of genomic integration of linear plasmids compared to wildtype cells. None of the cell lines tested appeared to have a lowered transfection efficiency (Harrington, 1992; Manivasakam, 2001; Willers, 2002; Zhang, 2004). In the case of the Nbs1-mutant ES cell line, expression of an *Nbs1* transgene restored the efficiency of plasmid integration (Zhang, 2004). For each of the genes listed above, the cell line (or mouse model) has a V(D)J recombination

deficiency (or is immunodeficient) and shows hypersensitivity to DSB-inducing agents, indicating that lack of these genes correlates with repair-related and V(D)J-related NHEJ (Biedermann, 1991; Pergola, 1993; Errami, 1998; Gao, 2000; Kang, 2002).

NHEJ in *gol/gol* ES cells was assessed by integration of a selectable cassette into the genome. Data reported in this chapter suggest that *gol/gol* ES cells have an elevated efficiency of NHEJ compared to wildtype cells, which agrees with several previous studies The majority of studies using the human HCC1937 cell line showed that these cells do not appear to have a defect in NHEJ (Table 1.5). A recent experiment done using HCC1937 cells gave results very similar to the ones generated in this study: plasmid integration in HCC1937 cells was increased compared to wildtype cells. Addition of a *BRCA1* transgene to the cells brought the integration frequency back to wildtype levels (Zhang, 2004). In the *Brca1*$^{\Delta X.11/\Delta X.11}$ murine ES cells studied by Moynahan *et al*., the efficiency of NHEJ was elevated. This elevation was corrected by addition of a *Brca1* transgene, indicating that the change was likely due to the loss of *Brca1* (Moynahan, 1999; Snouwaert, 1999). These same cells have been assayed for NHEJ using the I-SceI assay. The result of the two assays, while not identical in fold-difference, show the same general trend of increase or decrease (Moynahan, 1999; Moynahan, 2001; Zhong, 2002a).

In contrast to the results in this chapter, several experiments have indicated that *Brca1*$^{-/-}$*, p53*$^{-/-}$ MEFs have a lower efficiency of NHEJ as compared to that of *p53*$^{-/-}$ MEFs (allele described in Table 1.3 #4) (Zhong, 2002a; Zhong, 2002b). The reason for this difference is not clear, but some of the assays performed on these MEFs indicate that they may have a defect in precise end joining but not in overall end-joining. This observation is supported by a recent study which showed that lymphoblastic cell lines generated from breast-cancer patients who carry a *BRCA1* mutation have a deficiency in precise end-joining, although their overall end-joining is similar to that of lymphoblast cell lines derived from healthy control individuals (Baldeyron, 2002). However, there is a possibility that the *Brca1*$^{-/-}$*, p53*$^{-/-}$ MEF line

carries other mutations, and that the cell lines derived from adult cancer patients may also have secondary mutations. It is difficult to compare the results generated using *gol/gol* ES cells with data from *Brca1*$^{-/-}$, *p53*$^{-/-}$ MEFs, as *gol/gol* cells were assessed only for the efficiency of overall end-joining, not for the precision with which they repair a break.

5.3.2.3 gol/gol cells: difference between cell lines

Data presented in Figures 5.7 and 5.8 indicates that the *gol/gol* ES cell lines perform consistently between assays, but they are noticeably different when compared to one another. This may indicate that there are secondary mutations in one cell line vs. the other, and perhaps the use of SKY, chromosome banding, or CGH array (readily available in the lab) would be of use in assaying for any gross rearrangements in the genome of these cell lines. The use of other assays, such as the I-SceI assay or PFGE is desirable and should highlight if the cell-line difference is a real effect or not. The only problem that might arise from use of the I-SceI assay (although a substrate might be designed to get around this problem) is that if accurate NHEJ is elevated in the *gol/gol* cells and HRR is depressed, NHEJ events might be scored as HRR events and lead to wildtype and mutant cells having little overall difference. This concern may be unfounded, as the accuracy of NHEJ in *gol/gol* cells has not been measured. PFGE-based monitoring of break repair following damage might also be used. Since the change in the kinetics of damage repair can be used to determine which form of DSBR is non-functional, this assay (done over a range of doses) might more clearly define the repair defects of the *gol/gol* cells. Before doing either of these assays, it would be useful to look at the cell-cycle kinetics of *gol/gol* ES cells following DSB-related DNA damage; the results of such a study should indicate if blockage/loss of checkpoint control occurs following damage.

5.3.2.4 In summary

The elevation of NHEJ efficiency and slight decrease in HRR efficiency in *gol/gol* cells may describe a mechanism by which Brca1 contributes to tumourigenesis: an increase in error-prone repair coupled to a backlog of unrepaired lesions. This idea correlates well with the expression levels of

Brca1; it is a protein expressed maximally in S and G2/M phases, phases in which HRR is thought to be the major repair pathway (Ruffner and Verma, 1997; Scully, 1997b). Whether this means that the *gol/gol* ES cells have a defect in normal or break-induced cell cycle phase distribution has yet to be determined. The question of why an increase in NHEJ would not phenotypically "rescue" the HRR deficiency is answered by several studies indicating that the two forms of repair do not complement one another. A previous study using mice deficient for either *Rad54* (involved in HRR) or the gene encoding DNA-PKcs (*scid*, essential for NHEJ) demonstrated that *Rad54*$^{-/-}$, *scid*$^{-/-}$ double knockout mice were more hypersensitive to γ-irradiation than mice deficient for only one gene (Essers, 2000). Additionally, *Rad54*$^{-/-}$ or *Ku70*$^{-/-}$ (NHEJ deficient) chicken DT40 cells have been used to demonstrate that *Rad54*$^{-/-}$ cells are γ-irradiation sensitive in late S and G2 phases, while *Ku70*$^{-/-}$ cells are sensitive to γ-irradiation in G1 and early S phases. Cells lacking both genes are more sensitive to γ-irradiation than cells lacking only one gene (Takata, 1998; Wang, 2001b). While the major defect in *gol/gol* cells appears to involve NHEJ, their small decrease in HRR efficiency may also be significant. Previous studies using *scid*$^{-/-}$ fibroblasts have shown that these cells are not hypersensitive to MMC, while cells lacking *Rad54* (and *gol/gol* cells) are hypersensitive to MMC (Biedermann, 1991; Hendrickson, 1991; Essers, 2000). This suggests that at least some of the phenotypes observed in *gol/gol* cells may be attributable to their HRR deficiency.

Experiments described in this chapter also confirm the previous observation that while human tumour cell lines heterozygous for a *BRCA1* mutation appear to be more susceptible to γ-irradiation or MMC than wildtype cell lines, mouse heterozygous-mutant cell lines are not (Figures 5.4, 5.5 (Abbott, 1999; Foray, 1999; Moynahan, 2001)). It is possible to take this observation as an argument that human and murine carriers of *BRCA1* mutations have different cancer predispositions or that human *BRCA1* is a haploinsufficient gene. Perhaps a more logical conclusion is that studies of human tumour cell lines heterozygous for *BRCA1* mutations may be affected by other mutations

carried by these cells, although some human *BRCA1* mutations may well be haploinsufficient.

5.3.3 Immunolocalization of Brca1^{gol}

Immunolocalization studies indicate that Brca1^{gol} is able to localize to the nucleus and appears to form S phase nuclear foci. These results have implications for the interaction of Brca1^{gol} with other proteins, as well as on its biology, which will be discussed further in Chapter 6. However, the level of cytoplasmic localization of Brca1, while consistent between the wildtype and mutant cell lines, is higher than is generally seen, and the overall pattern is more punctate than is generally seen. In order to rule out non-specificity of the antibody being mistake for cytoplasmic localization, the immunolocalization experiments should be redone either with a different antibody, or in conjunction with a peptide which acts as competitor for the antibody.

Following both γ-irradiation and UV exposure, a similar percentage of both wildtype and *gol/gol* cells exhibit Brca1 nuclear foci (Table 5.1). As nuclear foci are also observed in undamaged *gol/gol* cells, this suggests that the N-terminal domain predicted to be missing in Brca1^{gol} is not required for the formation of either normal or damage-induced nuclear foci. However, the amount of focus formation may be lower than it appears, as the overall punctate appearance of the immunolocalization pattern may lead to an overestimation of foci formation. Previously, it was shown that damage-induced foci will form in MEFs carrying the ΔX.11 form of Brca1, but that a version of the human BRCA1 protein lacking part of the C-terminus does not form such foci (Zhong, 1999; Wu, 2000; Huber, 2001). Taken together with data generated from *gol/gol* cells, this suggests that only the C-terminus may be required for the formation of nuclear foci. However, this hypothesis is contradicted by the results of Chiba and Parvin, who overexpressed a version of the human BRCA1 protein lacking residues 1-302 (lacking the RING domain and the NES) and found it did not form normal S phase foci. Additionally, when they overexpressed a mutant form of BRCA1 lacking the

C-terminal BRCT repeats, S phase BRCA1 nuclear foci were observed (Chiba and Parvin, 2002). It is possible that overexpression of the mutant proteins in their study may have resulted in aberrant localization or incorrect protein folding, leading to the discrepancy between these results and the others described above.

Hyperphosphorylation of BRCA1 following DNA damage appears to be the major impetus for dispersion of the protein from normal S phase foci and re-formation into damage-induced foci. While the phosphorylation status of Brca1^{gol} was not investigated, all known phosphorylation sites in the Brca1 protein are downstream of the region deleted by the *gol* allele and are expected to be intact in the Brca1^{gol} protein, suggesting that regulation by this mechanism would still occur (Rosen, 2003). This supposition is supported by the fact that *gol/gol* cells appear to exhibit both S phase and damage-induced nuclear foci in a percentage of cells observed (Figures 5.9 – 5.12, Table 5.1).

A percentage of both non-treated and γ-irradiated *gol/gol* cells have large aggregates of protein, which are rarely seen in γ-irradiated wildtype cells, or in UV-treated wildtype or *gol/gol* cells (inset Figure 5.12 and Table 5.1). The presence of protein aggregates in *gol/gol* cells may indicate that Brca1^{gol} is overexpressed, less soluble, or more stable compared to wildtype Brca1. The stability/overexpression of the Brca1^{gol} protein will be discussed further in Chapter 6. Although aggregates are observed in some γ-irradiated wildtype cells, they are not observed as frequently (Table 5.1). Perinuclear localization of a subtype of phosphorylated BRCA1 has been reported by one group (following UV or γ-irradiation-induced DNA damage in the human MCF7 cell line) (Okada and Ouchi, 2003), and perinuclear concentration of BRCA1 in a human breast cancer cell line has been seen (De Potter, 1998). However, these are not commonly reported findings, and aggregates not been reported previously in studies of Brca1 immunolocalization, including one in which the same antibody was used (Bachelier, 2000). This suggests that they are not likely to be an antibody artifact. They could be a result of using randomly-differentiated cells (which have also not been used in previous studies). To address these concerns, it would be highly desirable to use *gol/gol* MEFs (if

they can be generated) in these studies instead of the differentiated cells, as well as an additional antibody or peptide-competition in conjunction with the M-20 antibody.

5.3.4 Summary

The use of *gol/gol* ES cells has helped to further define the role of Brca1 in the response to DNA damage. Data generated using these cells support several previous observations, help to clarify the role of Brca1 in NHEJ, and indicate that the N-terminus of Brca1 may not be required for base repair or the formation of normal or damage-induced nuclear foci. The molecular characterization of the *gol* allele will be discussed in Chapter 6.

CHAPTER SIX:
MOLECULAR CHARACTERIZATION OF THE *gol* ALLELE OF *Brca1*

6.1 INTRODUCTION

The two *Brca1* deletion alleles generated in this study, *gol* and *Brca1*$^{-}$, are phenotypically different despite their structural similarity. Data presented in previous chapters demonstrated that neither *Brca1*$^{-/-}$ ES cells nor mice could be generated, but that *gol/gol* ES cells are viable and grow normally, although they are hypersensitive to DNA damage. The Brca1^{gol} protein is able to localize to the nucleus and is detected in nuclear foci both before and after DNA damage.

The final goal of this project was to investigate the consequences of the *gol* and *Brca1*$^{-}$ mutations on the regulation of Brca1, with a view toward explaining the difference in viability between the two alleles. Both were designed to replace exon 2 with selection cassettes transcribed in the opposite direction from the transcriptional orientation of *Brca1*. Aside from carrying different selection markers, the two alleles differ only in the amount of intronic sequence they delete: ~2 kb more genomic sequence is deleted in the *gol* allele (Figure 6.1).

6.1.1 Role of the PGK promoter

Initially it was hypothesized that the *gol* allele gave rise to an alternative *Brca1* transcript, driven by the PGK promoter from the *Puro* selection cassette. This promoter is reversed in relation to the direction of *Brca1* transcription, but previous reports have suggested that the PGK promoter may be able to act bidirectionally (Johnson and Friedmann, 1990; Abeliovich, 1992; Scacheri, 2001). As exon 2 of *Brca1* contains the translational start site, translation of an alternative transcript is likely to initiate from a downstream, in-frame AUG codon, of which there are three near the 5' end of *Brca1*. Experiments described in this chapter indicate that while the PGK promoter does appear to be able to act bidirectionally in ES cells, it does not appear to be affecting transcription of *Brca1* from the *gol* allele. Furthermore, while different isoforms of *Brca1* are expressed from the *gol* allele (the most prominent being the *Brca1 ΔX.2* isoform, described in Chapter 3, in which exon 1 is spliced

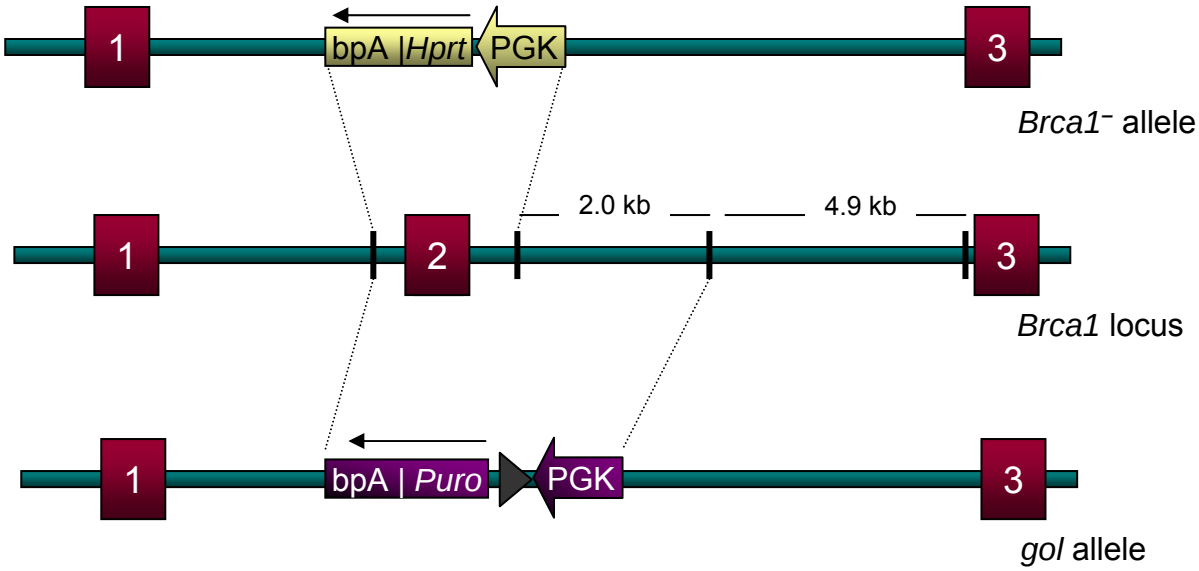


Figure 6.1: The *Brca1*⁻ and *gol* alleles (5' end).
The *Brca1*⁻ and *gol* alleles have two major differences: their selection cassettes, and the amount of intronic sequence they replace. ~2 kb more intronic sequence is deleted in the *gol* allele.
The grey triangle represents a *loxP* site.
Not drawn to scale.

directly to exon 3), no transcripts unique to the *gol* allele were detected by several different assays. This suggests that if a non-endogenous promoter is involved in the transcription of *Brca1*, the level of the novel transcript is too low to be detected by the methods used in this study.

6.1.2 Transcriptional control of the *Brca1*$^{-}$ and *gol* alleles

Although *Brca1* mRNA does not seem to be overexpressed in cells carrying a *gol* allele, the ratio of mutant-to-wildtype *Brca1* transcripts differs between *+/gol* and *+/–* ES cells, and Brca1 protein is more abundant in cells carrying a *gol* allele. This increase in protein level (or stability) supports the idea that translation of Brca1 initiates at a downstream AUG codon. The lack of a noticeable change in the *Brca1* mRNA level in cells carrying a *gol* allele may be due to overall regulation of *Brca1* levels in the cell. While there would then be no difference in the type of transcript (*Brca1 ΔX.2*) transcribed from *gol* or *Brca1*$^{-}$, the inviability of *–/–* ES cells and embryos (and viability of *gol/gol* ES cells) generated in this study suggests that the relative amount of transcription from *gol* is higher than from *Brca1*$^{-}$. It is hypothesized that the extra 2 kb of intronic sequence deleted in the *gol* allele may carry a transcriptional suppressor. An alternative hypothesis is that the *Puro* selection cassette carried by the *gol* allele serves as a transcriptional enhancer.

6.1.3 The interaction of Brca1^{gol} and Bard1

Bard1 is a RING-domain binding partner of Brca1 (Wu, 1996). As discussed earlier, the heterodimerization of Brca1 and Bard1 is thought to be of major importance in nuclear import, nuclear retention, and functionality of Brca1 (Fabbro, 2002). The interaction of Bard1 and Brca1^{gol} was assessed by co-immunoprecipitation. Bard1 appears to have a reduced amount of interaction with Brca1^{gol}, which extends the current knowledge about the dependence of Brca1 on its interaction with Bard1 for functionality. Furthermore, this result supports the supposition that the Brca1^{gol} protein does not have a fully-functional RING domain, as is predicted based on sequence and structure analyses of the *gol* allele.

6.2 RESULTS

6.2.1 Brca1^{gol}: predicted translation initiation sites

Exon 2 of *Brca1* encodes both the translational start site and part of the highly-conserved N-terminal RING domain. This domain is known to be important for protein-protein interactions, and is where Brca1 interacts with Bard1, its binding partner and putative nuclear chaperone (Wu, 1996). A look at the protein structure of the entwined RING domains of human BRCA1 and BARD1 (Figure 6.2a) shows that the region encoded by exon 2 (Figure 6.2b and c) is an integral part of the interaction structure. Indeed, *in vitro* studies have established that some point mutations in the RING domain of BRCA1 abolish its ability to heterodimerize with BARD1 (Wu, 1996; Brzovic, 1998; Brzovic, 2001a; Joukov, 2001b; Morris, 2002).

The viability of *gol/gol* ES cells suggests that the *gol* allele gives rise to a functional protein. As the translational initiation site of *Brca1* has been deleted in the *gol* transcript, and no AUG codons are present in exon 1, translation of this protein may initiate from an in-frame, downstream AUG codon. Three such AUG codons exist near the 5' end of *Brca1*, in exons 5 and 6 (Figure 6.3).

A favored model of eukaryotic translational initiation is the scanning model, whereby a ribosome binds to the 5' end of a message and scans in a 5'–3' direction until an AUG codon is encountered (Kozak, 1978; Kozak, 1989; Kozak, 1997). However, the first in-frame AUG encountered downstream from the exon 2 deletion is situated just upstream of the NES described in section 1.7, meaning that the Brca1^{gol} protein would lack the RING domain but have both an NES and NLSs. Experiments reported by Fabbro *et al.* using transiently expressed BRCA1 and BARD1 proteins suggest that a BRCA1 protein which lacks the RING domain but possesses a functional NES will be located mainly in the cytoplasm (Fabbro, 2002). This directly contradicts experimental results reported in Chapter 5 which showed that the localization of Brca1 in *gol/gol* and wildtype cells is very similar (Figure 5.9). Additionally,

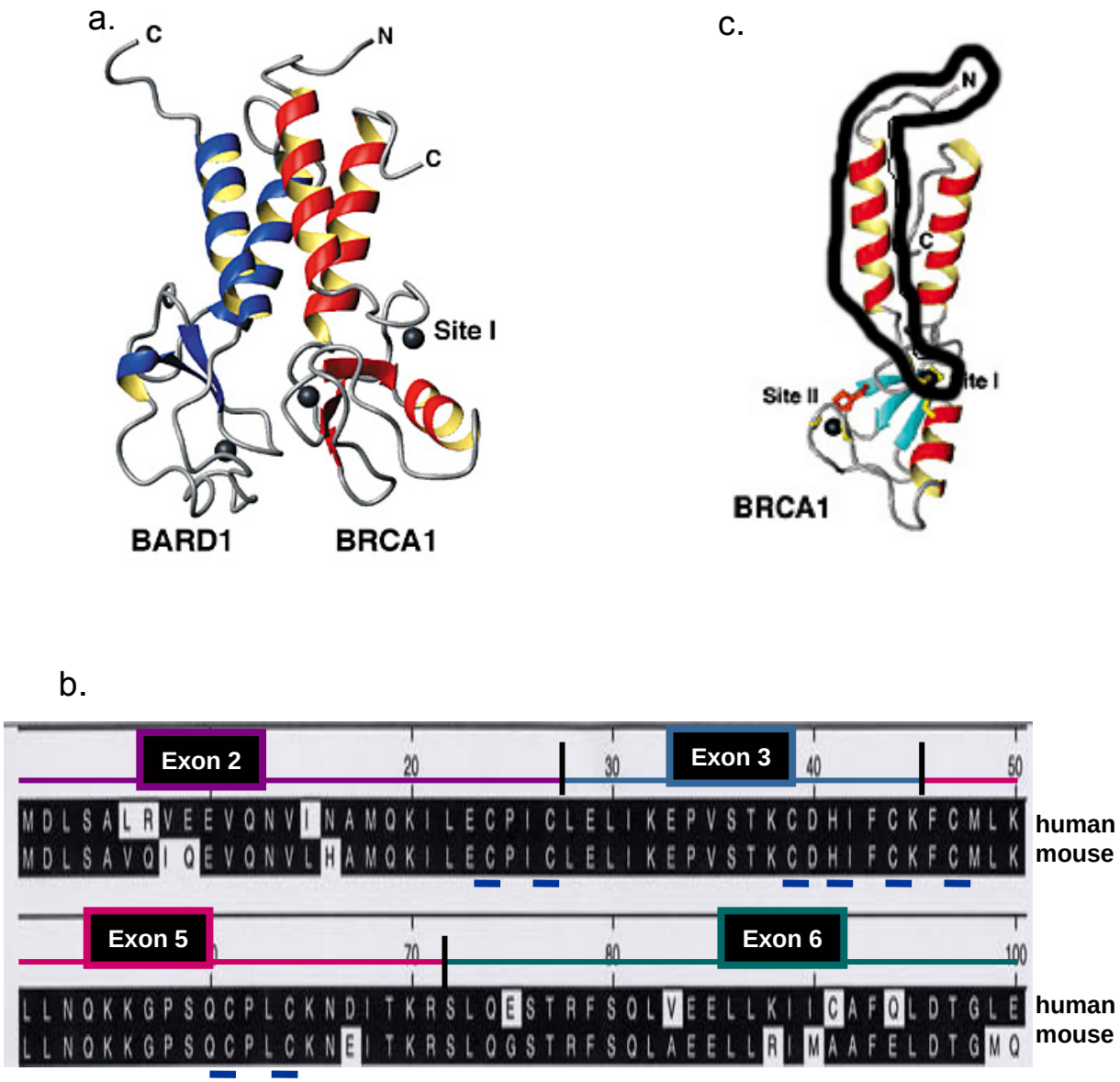
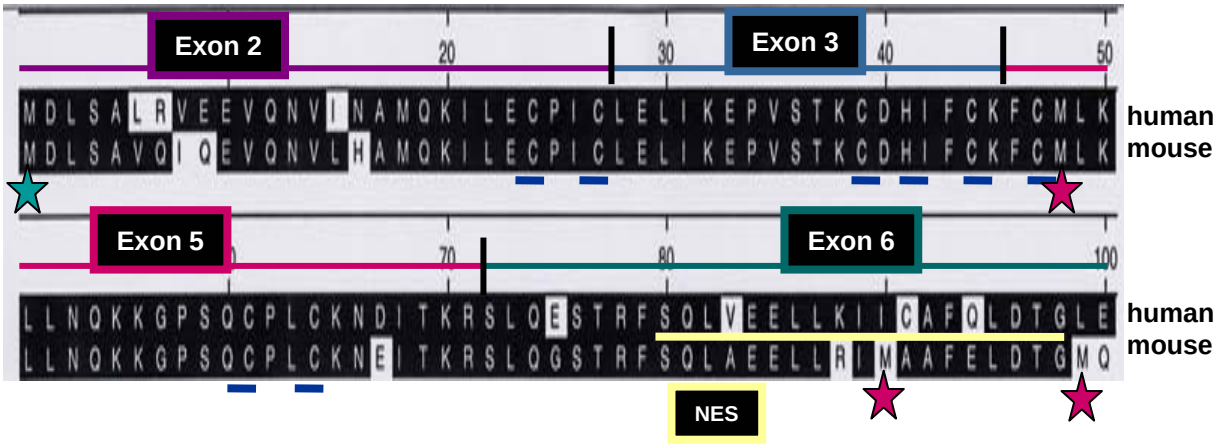


Figure 6.2: Structure of the BRCA1/BARD1 RING domain interaction.
a. Structure of the human BRCA1/BARD1 RING-RING interaction structure. **b.** Mouse-human Brca1 protein alignment including the first 100 residues of Brca1. Black highlighting indicates identical residues. Exon regions are indicated and the key cysteine (C) and histidine (H) residues of the Zn-finger RING motif are underlined in blue. **c.** The region encoded by exon 2 is circled on the BRCA1 structure.
Structural diagrams taken from (Brzovic, 2001).

a.



b.

Kozak:	GCC	PuCC	AUG	G
exon 2:	GAA	GAA	AUG	GAU
exon 5:	UUU	UGU	AUG	CUG
exon 6:	GAA	AUA	AUG	GCU
exon 6:	ACG	GGA	AUG	CAG

Figure 6.3: In-frame, 5' AUG codons of *Brca1*. a. Human-mouse Brca1 RING domain alignment, as in Figure 6.5. Red stars indicate potential downstream translational start sites, a green star denotes the normal start site. Exons and the nuclear export signal (NES) are indicated. **b.** Contextual setting of the endogenous and downstream AUG codons compared to the ideal Kozak consensus sequence. Bases in the +4 and -3 positions considered ideal in the Kozak model are underlined.

many experiments by Marilyn Kozak demonstrate that translational efficiency is highly influenced by the context of the AUG start codon, especially by the bases in the -3 and +4 positions (relative to the A of AUG) (Kozak, 1981). The ideal configuration, as determined both by experimentation and cataloguing of archived sequences, is a purine at -3 and a guanine (G) at +4 (Kozak, 1981; Kozak, 1984; Kozak, 2001). Similar experiments have suggested that if the first AUG encountered by a ribosome is in a poor context, it may not be recognized as a start site, and the ribosome will continue scanning (Kozak, 1978; Kozak, 1986).

Figure 6.3b shows the contextual setting of the downstream AUG codons of *Brca1*. The first AUG, in exon 5, is in a very poor context and *in vitro* experiments done by Kozak strongly suggest that it would be used rarely, if at all, as a start codon (Kozak, 1997). The second AUG codon, in exon 6, has a much more favorable context. Furthermore, this second AUG is located in the middle of the NES. If translation began from this site, the resulting Brca1^{gol} protein would lack both the RING domain and the NES, and, according to data reported by Fabbro *et al.*, would be able to localize to the nucleus using the NLSs coded by exon 11 (Fabbro, 2002). The use of the second AUG codon would correlate well with the immunolocalization data presented in Chapter 5. It is possible that translation initiates from more than one start site, possibly generating two forms of the protein with different cellular localizations. There is additionally the possibility that alternative start codons (codons which differ from AUG by one base, such as ACG or CUG) may be used for initiation (Touriol, 2003). The use of alternative start codons is not common, and several studies have indicated that the context of these sites is very important in determining how effectively they work (Mehdi, 1990; Boeck and Kolakofsky, 1994). Analysis of the *Brca1* sequence indicates that none of the first in-frame occurrences of alternative start codons occurs in a good context, suggesting that if they are used, the protein translated from them would probably be expressed at a low level. In this study, no experiments were performed to determine the start codon used in Brca1^{gol}.

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While downstream translational initiation of Brca1^{gol} seems likely – not least because *gol/gol* ES cells are viable – downstream initiation might be equally likely for transcripts of the *Brca1*$^{-}$ allele. That these two alleles are not phenotypically identical suggests that either translation from a downstream AUG in the *Brca1*$^{-}$ transcript is less robust, or that the *gol* allele produces a different transcript than does the *Brca1*$^{-}$ allele. In the case of the *gol* allele, this hypothetical different transcript could be expressed under the control of the PGK promoter from the *Puro* selection cassette, reversed in relation to the direction of *Brca1* transcription (see Figure 6.1). A reversed PGK promoter has been shown to drive expression of a reporter gene *in vitro* (albeit at low efficiency), and a few instances of aberrant transcription arising from a reversed PGK promoter have been reported in the literature (Johnson and Friedmann, 1990; Abeliovich, 1992; Scacheri, 2001).

Brca1^{-} also carries a reversed PGK promoter as part of its *Hprt* selection cassette (see Figure 6.1). The difference between these two promoters is their proximity to exon 3 – the *Puro* selection cassette is ~2 kb closer to exon 3 than is the *Hprt* cassette (Figure 6.1). Based on evidence described in Chapters 3 and 4, as well as data reported by Ludwig *et al.* (from whom the derivative of the *Brca1*-Hprt-TV was obtained), the *Brca1*$^{-}$ allele behaves as a null allele – that is, homozygous mutant embryos or ES cells are not viable (Hakem, 1996; Liu, 1996; Ludwig, 1997). This suggests that the level of transcription of the *Brca1*$^{-}$ allele driven by the reversed PGK promoter is negligible at best. If the position of the reversed PGK promoter is important for expression of *gol*, this hints that intronic sequence near the PGK promoter in the *gol* allele may be important for expression, that perhaps the promoter is using nearby intronic sequence as an alternative transcriptional start site.

6.2.2 PGK is a bidirectional promoter in ES cells

Some evidence generated from *in vivo* expression and *in vitro* reporter-gene experiments suggest that the bidirectional activity of PGK may be cell-, tissue-, or gene-specific (Johnson and Friedmann, 1990; Abeliovich, 1992). To test if the reversed PGK promoter is effective in ES cells, three *Puro*

vectors (*Puro* expressed using either the PGK promoter, a reversed PGK promoter, or no promoter) were electroporated into wildtype ES cells which were then selected in puro-containing medium. Table 6.1 shows that the reversed PGK promoter does appear to be able to drive expression of a reporter gene in ES cells, albeit at a lower level than the control promoter (~15%). This compared favorably with a previously-reported *in vitro* study, in which a reporter gene driven by the reversed PGK promoter was expressed at ~10% of the normal level (Johnson and Friedmann, 1990).

As electroporation generally results in tandem integration, puro-resistant colonies could result from integration of the revPGK-*Puro* cassette if read-through occurred between adjacent copies. However, while a tail-to-tail integration would provide such a read-through substrate, the PGK promoter would have to traverse two ampicillin-resistance cassettes to reach the reporter gene, making this an unlikely event (Figure 6.4).

While this experiment indicated that the PGK promoter appears to be able to act bidirectionally in ES cells, an added layer of complexity is present in the *gol* and *Brca1*$^{-}$ alleles, as the promoter is also driving transcription of a gene in the forward direction. Only one *in vitro* dual-reporter gene experiment has been reported in the literature; it suggested the possibility of decreased expression of the reporter construct in the reverse direction when a second gene was being transcribed in the forward direction (Johnson and Friedmann, 1990). However, if the PGK promoter proves instrumental in expression of *gol*, this question can be directly investigated *in vivo* using the *c2* and *gol* alleles; the *c2* allele has not yet undergone Cre-mediated recombination, so the reversed PGK promoter is not actively transcribing *Puro* (although this does not mean that it is not operational), while in the *gol* allele, *Puro* is actively being transcribed (compare Figure 3.3b and c). Of the few groups which have reported aberrant expression from a reversed PGK promoter, only two determined the sequence of the resulting transcript. Both groups found that part of the reversed promoter itself was included in the resulting transcript: 308 bp of promoter sequence was included in one case, while in the second study, which involved multiple transgenic lines, the amount of

Table 6.1: Efficiency of the reversed PGK promoter in ES cells.
Total puro-resistant colonies resulting from electroporation of plasmids carrying *Puro* driven by the PGK promoter (PGK), reversed PGK promoter (revPGK), or no promoter into wildtype ES cells.

Vector	Colonies experiment 1	Colonies experiment 2	Percentage of control experiment 1	Percentage of control experiment 2
PGK-*Puro*-bpA	1880	2592	100	100
revPGK-*Puro*-bpA	256	448	14	17
Puro-bpA	0	0	0	0

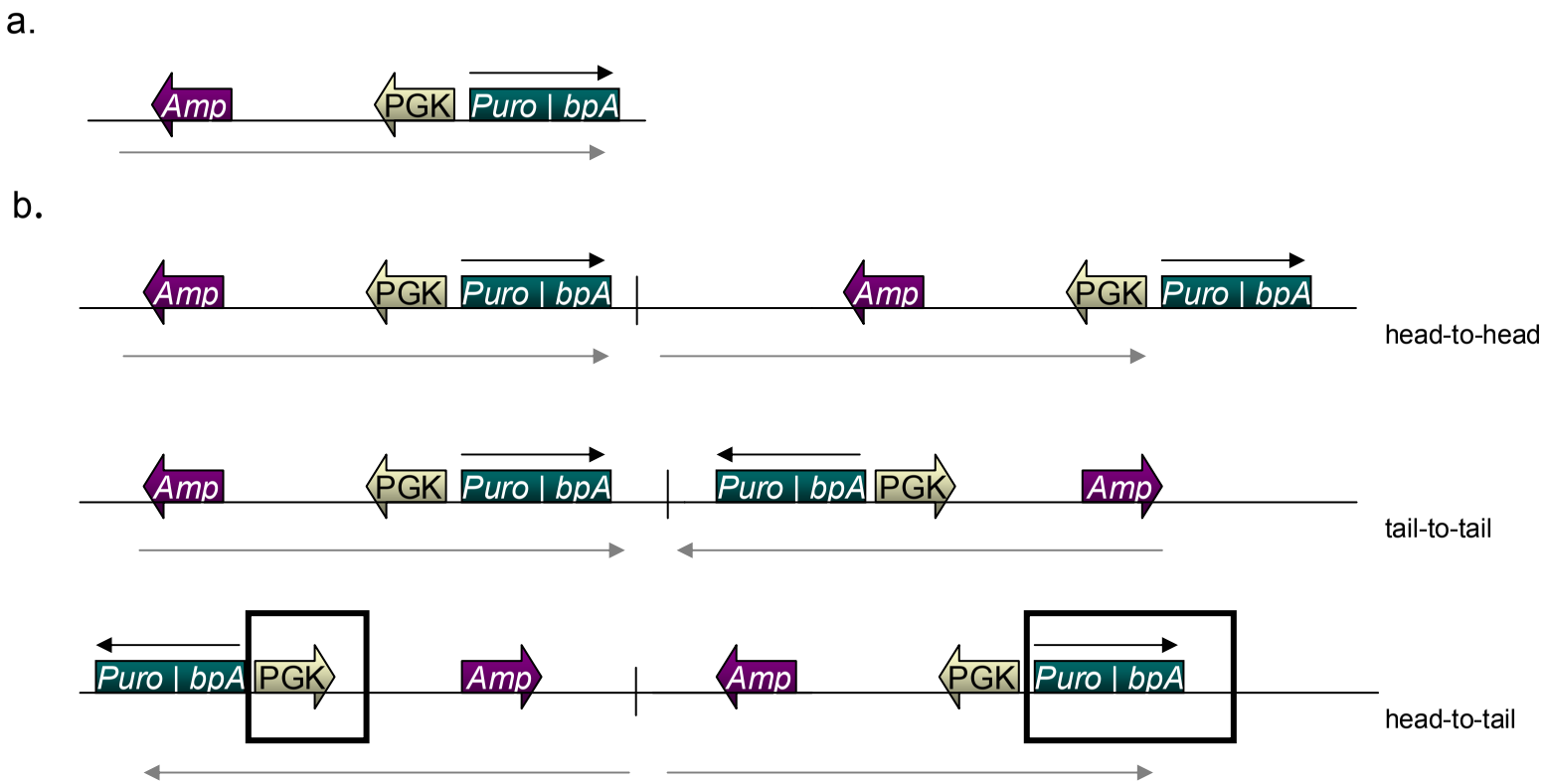


Figure 6.4: Possible orientation of tandem integrations of the revPGK-*Puro*-bpA plasmid.
a. The revPGK-*Puro*-bpA construct, linearized at the 3' end of the *Puro* gene. **b.** The three possible orientations of multiple insertions of the revPGK-*Puro*-bpA construct. Only a tail-to-tail integration forms a potential "read-through" PGK-*Puro*-bpA cassette (boxed), and two ampicillin-resistance (*Amp)* cassettes lie between promoter and coding region, making this an unlikely event.
Total vector size is 4.5 kb; the revPGK-*Puro*-bpA cassette is 1.5 kb. Figure is not drawn to scale.

promoter sequence varied (Abeliovich, 1992; Scacheri, 2001; Scacheri, 2003).

6.2.3 Multiple products are detected in a 5' RACE assay using *gol/gol* ES cell RNA

To try and identify transcripts expressed from the reversed PGK promoter, 5' Rapid Amplification of cDNA Ends (5' RACE), which utilizes known sequence from a transcript to amplify an unknown 5' region, was performed using total RNA from *gol/gol*, *+/–*, and wildtype ES cells. This analysis was expected to indicate the presence of an alternative transcript, lacking exon 1, present in *gol*-expressing cells but not in wildtype cells. Any putative alternative transcript might or might not be present in *+/–* cells, depending on the activity of the reversed PGK promoter in the *Brca1⁻* allele. 5' RACE was also expected to reveal if any intronic sequence was present in the putative PGK-driven transcript. Figure 6.5a shows that the predominant product amplified from *+/–* cells is a wildtype *Brca1* product. Two alternative products are also generated: the *ΔX.2 Brca1* isoform described in Chapter 3, in which exon 1 is spliced to exon 3, and an additional smaller product. *gol/gol* samples also show this smaller product, but their most abundant product is the *Brca1 ΔX.2* isoform – and no wildtype product is observed. A comparison of 5' RACE products from wildtype and *+/–* cells (Figure 6.5b) demonstrates that the wildtype *Brca1* allele also produces the smaller alternative product.

Sequence analysis of this smaller product indicates that it is a mix of two products, which end at the two AUG codons of exon 6 (Figure 6.5c). Random subcloning and sequencing of many 5' RACE products from *gol/gol* cells indicate that while other products are produced (including one which ends at the exon 5 AUG), the exon 6-AUG and *Brca1 ΔX.2* products are by far the most common. Analysis of a large number of subcloned *gol/gol* 5' RACE products did not identify any product containing sequence from either the PGK promoter or a *Brca1* intron (except for an intron 9-containing product which was also detected in wildtype samples; data not shown).

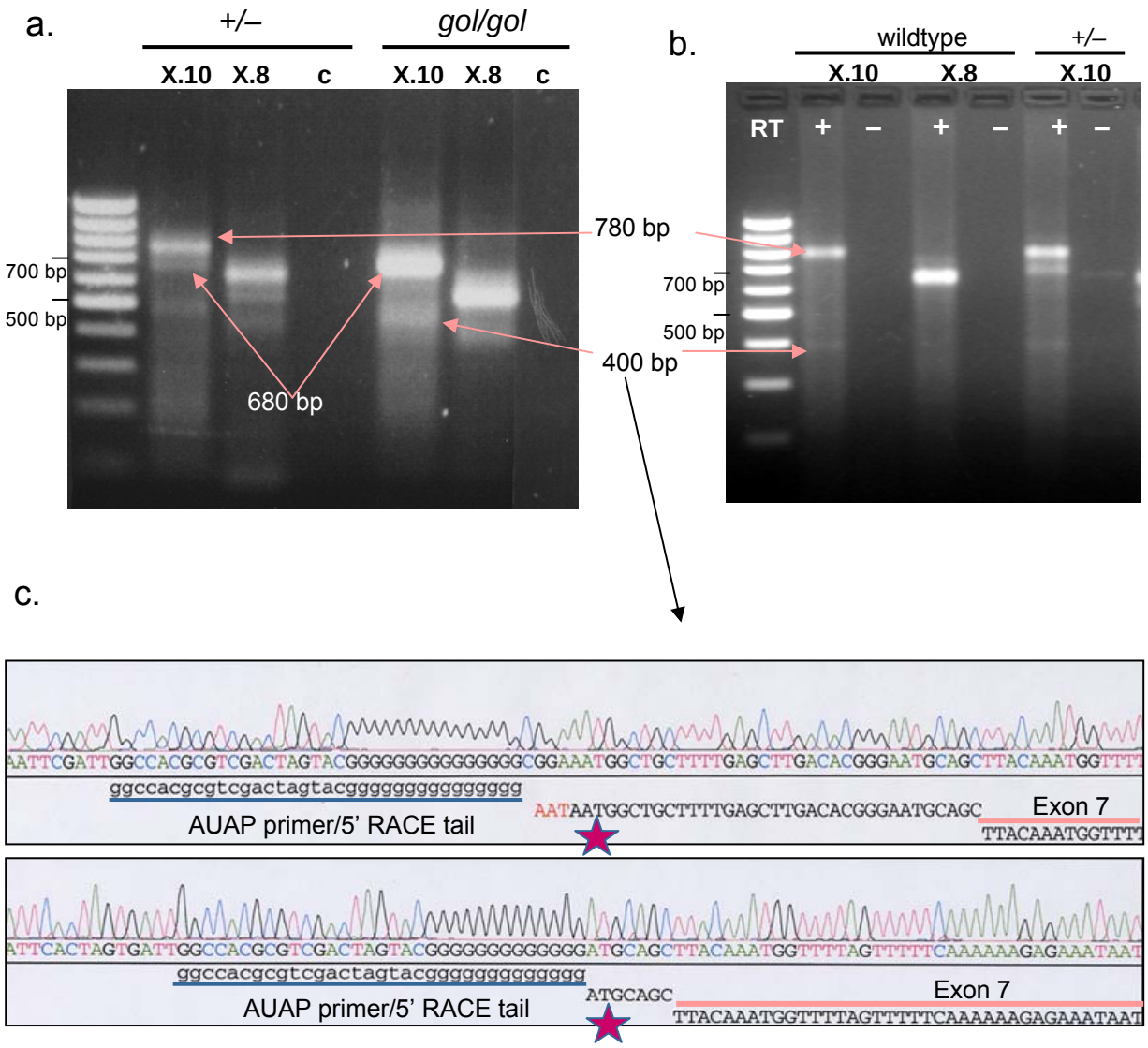


Figure 6.5: 5'RACE analysis of wildtype, +/–, and *gol/gol* ES cell RNA. **a.** 5' RACE of RNA from ES cells of indicated genotypes, followed by PCR with AAP and *Brca1* X.10R or *Brca1* X.8R2 primers. A variety of products are amplified from *gol/gol* and +/– samples. No unique products are observed in *gol/gol* samples. The 780 bp product is wildtype *Brca1*. **b.** 5' RACE followed by PCR (as in a.) using wildtype and +/- RNA samples. The smaller, 400 bp product also appears in wildtype *Brca1* X.10–amplified samples. **c.** Sequence analysis of the 400 bp band. A mix of two products is observed, each ending at one of the two exon 6 AUGs (red stars). "RT +" or "RT –" indicates if reverse transcriptase was added to the 5' RACE reaction or not. "c"=non-primer control PCR reaction, X=exon, AUAP=5'RACE tail sequence, AAP=Abridged Anchor Primer.

While 5' RACE did not reveal the presence of unique *Brca1* transcripts in *gol/gol* cells, the possibilities remained that such transcripts are expressed at a low level and were not detected, or that the alternative transcript starts further 3' than the exon 10 primer used for the 5' RACE reaction (see section 2.4.3 for sequence of the primer), and thus would not be detected by this experiment.

6.2.4 Detection of PGK sequences by Northern blot analysis

As two previous reports of aberrant transcripts expressed from a reversed PGK promoter have demonstrated that sequence from the promoter itself was included in the transcripts (Johnson and Friedmann, 1990; Abeliovich, 1992; Scacheri, 2001), potential inclusion of PGK promoter sequence in the *gol* transcript was tested by Northern blot.

A Northern blot of total RNA from wildtype and *gol/gol* ES cells was probed with a DNA probe comprising both strands of sequence from the first half of the PGK promoter (5'  3' – dark half). No transcripts were identified in any sample using this probe (data not shown). Hybridization and washing of this blot were done in tandem with a second Northern blot; successful hybridization of the second blot suggested that faulty technique did not account for the lack of signal. The blot used was subsequently and successfully hybridized with a control probe. RT-PCR analysis was also used to try to identify a potential PGK–*Brca1* fusion, using primers in the reversed PGK promoter paired with downstream *Brca1* reverse primers, but no such fusion transcript was identified (data not shown).

6.2.5 Direct sequencing of 5' RACE products

While no evidence for the use of a reversed PGK promoter was identified from subcloning and sequencing 5' RACE products, and the inclusion of the PGK promoter in a *gol* transcript is not supported by Northern blot or RT-PCR analyses, it was still possible that transcription is driven by the reversed PGK promoter but promoter sequence is not included in the resulting transcript.

Therefore, an attempt was made to identify a novel transcript driven by the PGK promoter by directly sequencing a pool of PCR-amplified 5' RACE products. By sequencing the entire pool of products, the presence of transcripts with novel 5' sequence should be indicated by an "interference pattern" in the sequence where the difference occurs. As a control, a pool of products generated using +/– RNA was used; a proportion of these transcripts lack exon 2, so a difference is expected at the exon 2–exon 3 boundary (inset, Figure 6.6). *gol/gol* and +/– RNA was subjected to 5' RACE and sequenced using several reverse *Brca1* primers.

As expected, sequence of the +/– 5' RACE product pool shows an "interference pattern" at exon 2 (Figure 6.6). No such pattern is seen in *gol/gol* cells at any point between exons 1 and 9 (Figure 6.7 shows two representative sequence trace files). A second 5' RACE reaction was run, but the results did not differ. It should be noted that *Brca1* does not have an exon 4, for historical reasons (see section 1.2.2). Taken in conjunction with the previous experiments, this suggested that the reversed PGK promoter does not play a major role in expression of the *gol* allele. The possibility still exists that expression is at too low a level to allow for detection by the above methods; but it is then questionable whether such a low level of expression would be biologically relevant. This experiment still does not exclude the possibility that the reversed PGK promoter drives expression of an alternative *Brca1* transcript which initiates further downstream than was tested by these 5' RACE reactions; but in that case, the alternative transcript should be at least 1 kb smaller than the wildtype transcript, and thus detectable on a Northern blot.

6.2.6 Analysis of *Brca1* expression : Northern blot and semi-quantitative RT-PCR

Northern blot analysis of *Brca1* expression in ES cells carrying the *gol* allele was performed using two cDNA probes; one containing exons 6-10, and another exons 22-24. This latter, 3' probe was expected to hybridize to both endogenous and alternative transcripts.

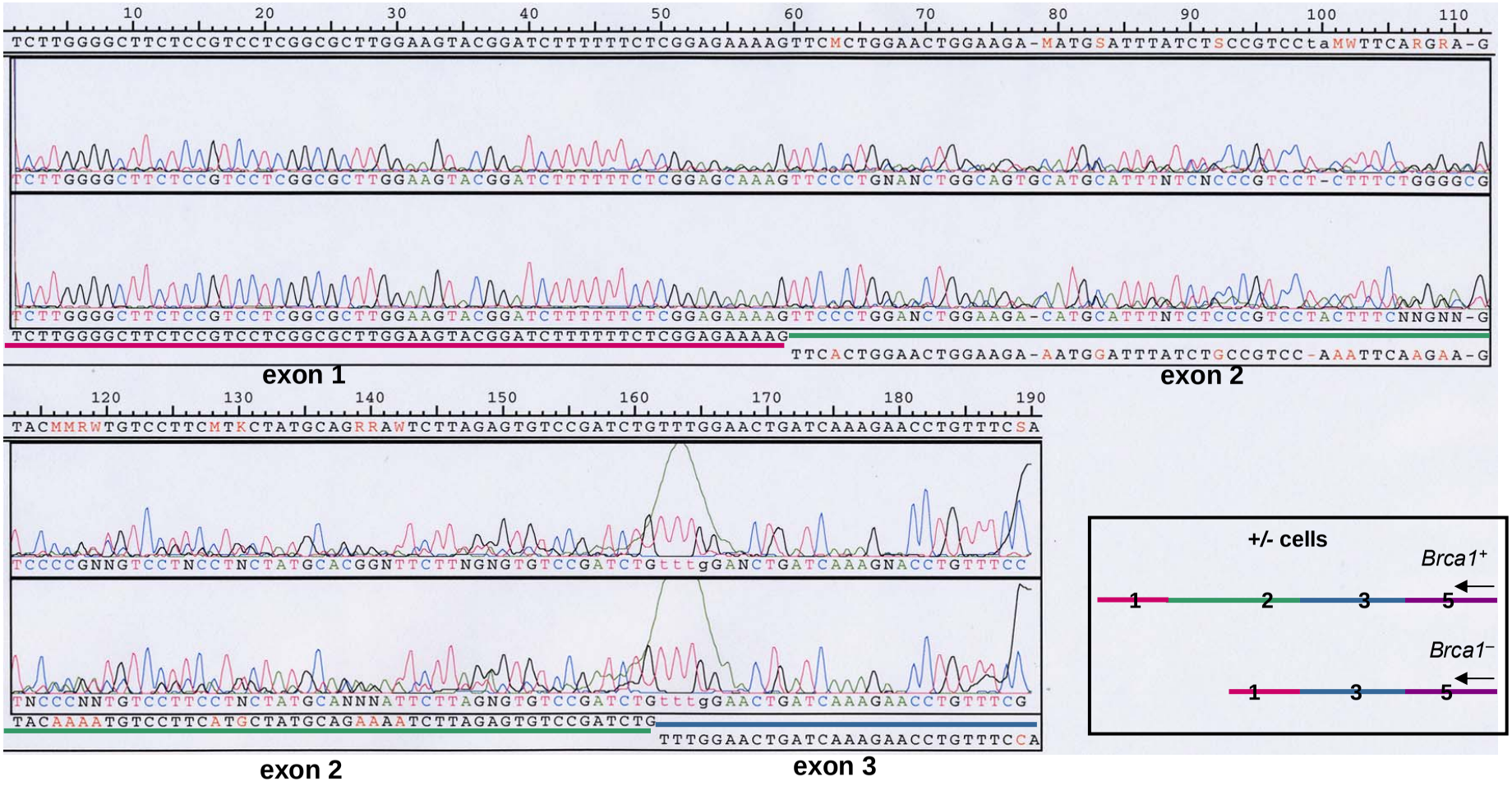


Figure 6.6: Direct sequencing of the +/– 5' RACE product pool. a. Sequence of pooled 5' RACE products from +/– ES cell RNA, showing an "interference pattern" at exon 2 (underlined in green). PCR amplification of 5' RACE pools was performed using the Abridged Anchor Primer (AAP) and *Brca1* exon 10R. Products shown here were sequenced using the *Brca1* exon 5 R primer. **Inset:** wildtype and *Brca1*$^{-}$ alleles. An interference pattern is expected to be evident where the two alleles do not overlap exactly (most of exon 2). *Brca1* does not have an exon 4.

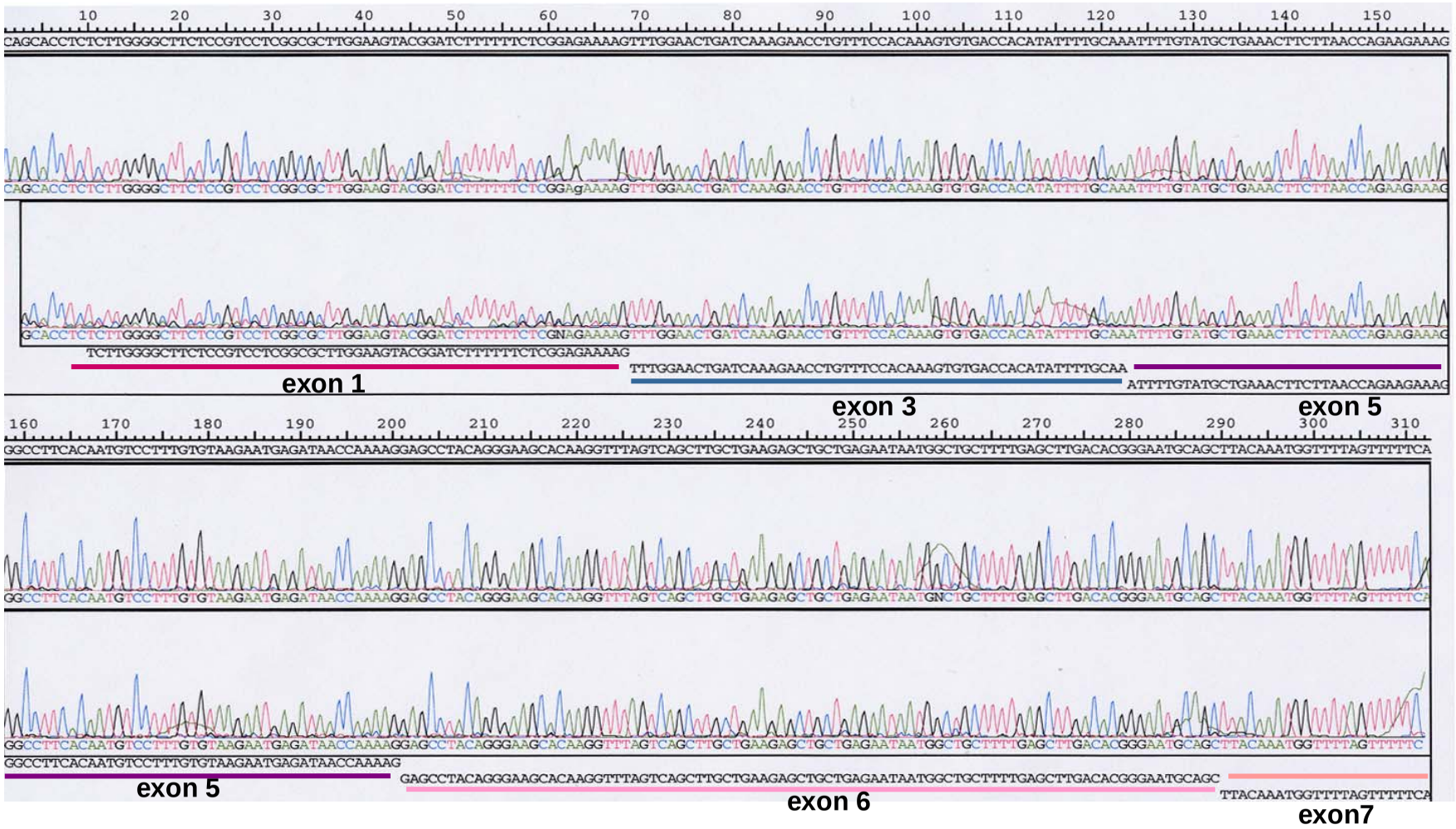
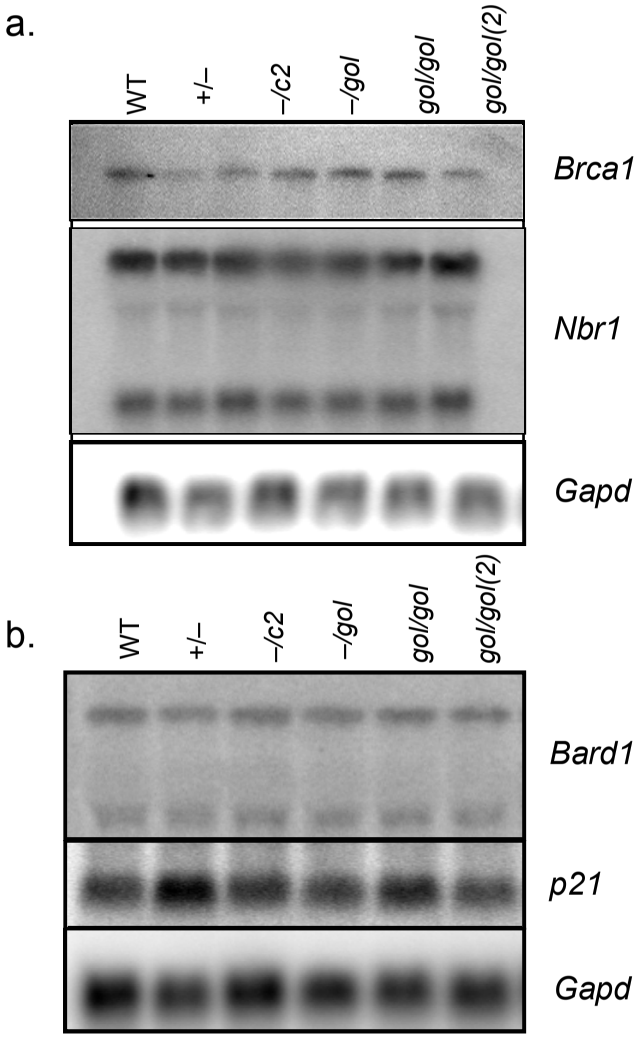


Figure 6.7: Direct sequencing of the *gol/gol* 5' RACE product pool. Sequence of pooled 5' RACE products amplified from *gol/gol* ES cell RNA. No interference pattern is seen at any point up to the exon 6-7 boundary. This sequence shows the exon 1-3 splice pattern of the major transcriptional product of the *gol* allele, *Brca1 ΔX.2*. PCR amplification of 5' RACE pools was performed using the Abridged Anchor Primer (AAP) and *Brca1* exon 10R. Products shown here were sequenced using the *Brca1* exon 7 R primer. *Brca1* does not have an exon 4.

The probe consisting of exons 6-10 appeared to cross-react with the rRNA bands, but only one *Brca1* transcript was detected (data not shown). The 3' probe detected only a ~7.4 kb transcript, corresponding to full-length *Brca1*, in all samples. The level of *Brca1* expression is not appreciably different between wildtype, *+/–,* or *gol/gol* cells after expression is normalized to the level of a *Gapd* loading control (Figure 6.8a and Table 6.2a). The normal *ΔX.11 Brca1* splice isoform was not detected by the 3' probe, although it is detected by RT-PCR (Figure 6.8c). Failure to detect this product by Northern blot may be because the overall level of *Brca1* expression was fairly low.

Semi-quantitative RT-PCR was performed to determine the differences, if any, between the levels of *ΔX.2* and wildtype *Brca1* transcripts in ES cells of various genotypes (Figure 6.9). This assay was performed by generating cDNA from 5 μg of total cellular RNA, making serial dilutions of the cDNA, and performing duplex PCRs using primers in *Brca1* (exons 1 and 6) and *Gapd* (control). Pilot experiments were run in the first instance to determine optimal primer concentrations, annealing temperature, and Mg-ion concentrations for the PCR reactions, then the amount of template and the number of cycles to be used was determined. At the primer concentrations used, neither product reached plateau phase (point at which the amount of PCR product is no longer increase exponentially) before 33 cycles, and reactions were thus run for 31 (at 1:40 and 1:80 dilutions) and 33 (at 1:40, 1:80, and 1:160 dilutions) cycles. Reactions were done in triplicate, and two separate RNA samples of each genotype were subjected to analysis. Figure 6.9a shows a representative gel of PCR products and controls from various genotypes. Figure 6.9b shows a graphical representation of the results from an averaging of all experiments.

Two points are evident: one, the overall amount of *Brca1* mRNA, as normalized to the *Gapd* loading control, is very similar across all genotypes. Second, while the amount of mutant (*ΔX.2*) transcript differs only slightly between the *+/gol* and *+/–* cell lines, the ratio of the mutant product to the wildtype product is significantly different between the two lines. The ratio of



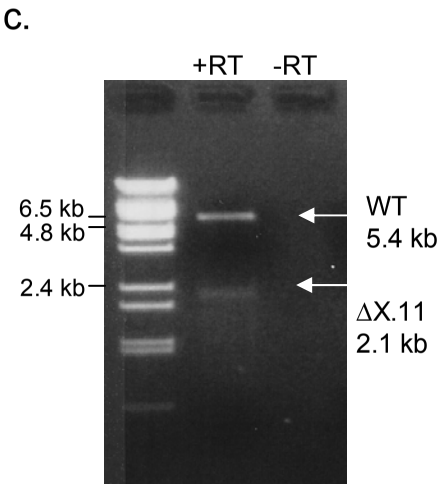


Figure 6.8: Expression levels of *Bard1, Brca1, p21, Nbr1*, and *Gapd* mRNAs. a. Northern blot of *Brca1* (3' cDNA probe comprising exons 22-24) and *Nbr1* expression compared to a *Gapd* loading control. **b.** Northern blot of *Bard1* and *p21* expression levels compared to a *Gapd* loading control. Probes were hybridized serially, after stripping, to the same blot. 20 μg of total ES cell RNA was loaded for each sample. **c.** RT-PCR of wildtype ES cell RNA, using PCR primers in *Brca1* exons 2 and 24. The smaller *ΔX.11 Brca1* isoform and full-length transcript are both detected.
WT=wildtype. See Table 6.2 for transcript quantitation.

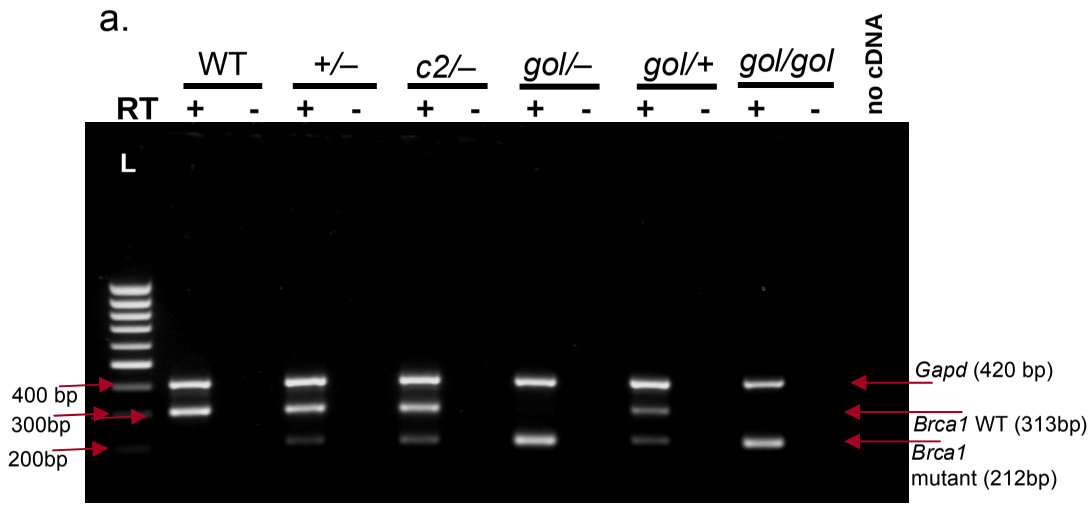
Table 6.2: Northern blot analysis: transcript intensities.
a. Total pixel volume for transcript-related bands from the Northern blot shown in Figure 6.2a. **b.** Total pixel volume for transcript-related bands from the Northern blot shown in Figure 6.2b. Pixel volume was measured in ImageQuant (Molecular Dynamics) with background subtracted. The relative volume of each transcript compared to a *Gapd* loading control is shown in the last two rows of each table. *Nbr1* and *Bard1* both have two transcripts; their values were averaged before being compared to the loading control.

a.

ES cell line:	wildtype	+/–	c2/–	–/gol	gol/gol	gol/gol(2)
Transcript						
Gapd	6.86	5.63	6.52	5.78	5.81	5.90
Nbr1 (average)	64.11	56.65	62.17	48.74	51.46	54.83
Brca1	3.98	2.96	3.37	3.88	4.01	3.93
Nbr1/Gapd	10.04	10.81	10.36	9.60	9.86	10.22
Brca1/Gapd	0.58	0.52	0.52	0.67	0.69	0.67

b.

ES cell line:	wildtype	+/–	c2/–	–/gol	gol/gol	gol/gol(2)
Transcript						
Gapd	7.48	6.47	7.61	6.83	6.71	7.06
Bard1 (average)	2.15	1.76	2.43	2.13	2.18	2.02
p21	13.61	19.90	14.90	12.92	15.64	11.94
Bard1/Gapd	0.29	0.27	0.32	0.31	0.33	0.29
p21/Gapd	1.82	3.08	1.96	1.89	2.33	1.69



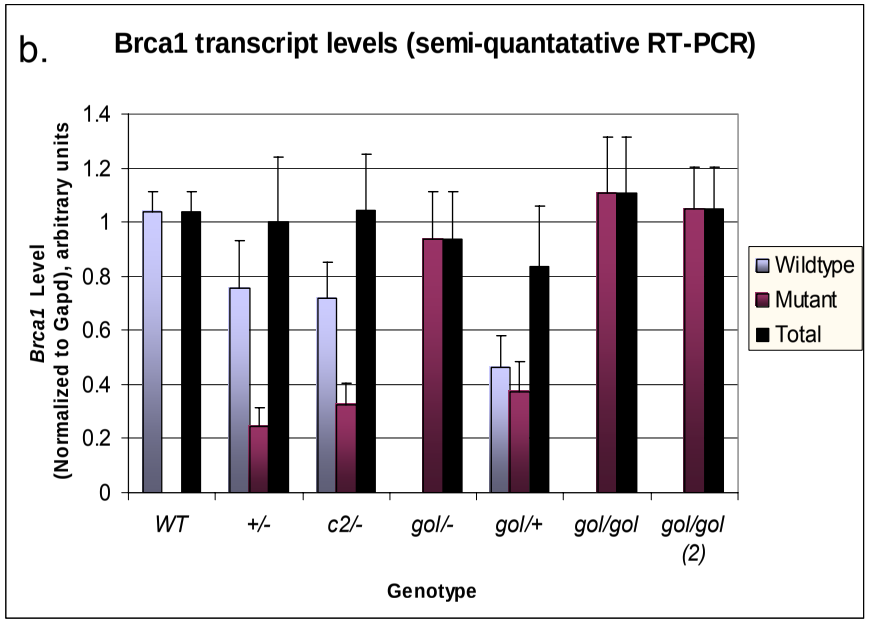


Figure 6.9: Semi-quantatative RT-PCR of *Brca1*.
a. Representative PCR gel of semi-quantataive PCR run on cDNA of the indicated genotypes. - RT indicates samples without reverse transcrptase added. See materials and methods for complete methods; this gel depicts samples from a 33-cycle PCR using PCR primers in *Brca1* exons 1 and 6 (lower two bands) and *Gapd* control primers (top band) using a 1:40 dilution of cDNA.
b. Gradphical representation of wildtype and mutant Brca1 levels (normalized to the Gapd control). The overall level of Brca1 is indicated by the black bar.
Error bars represent one standard deviation of all samples.
L=ladder, RT=reverse transcriptase.

mutant:wildtype product in *+/gol* cells is 1:1.2, while the ratio is 1:3.1 in *+/–* cells. This suggests that while there may be an overall control on the amount of *Brca1* transcript in cells (regardless of genotype), the ratio of the products appears to be the only real difference between the two mutant cell lines. This will be discussed further in light of data presented in the next sections.

6.2.7 Northern blot analysis of other genes

As no change in *Brca1* expression was observed between *gol/gol* and wildtype ES cells, the expression level of three other genes with known links to *Brca1* were assessed in *gol/gol* ES cells (for all three, transcript level was compared to that of a *Gapd* control). *Brca1* shares a bidirectional promoter with the gene *Neighbour of Brca1 1* (*Nbr1*); it was hypothesized that a change in the expression of this gene might contribute to or cause the phenotype of the *gol/gol* cells. However, the expression of this gene is not changed in *gol/gol* ES cells (Figure 6.8a and Table 6.2a).

In *Xenopus* (frog) embryos, following antisense-mediated depletion of *xBRCA1* (the *Xenopus* homologue of *BRCA1*), the level of xBARD1 (the *Xenopus* homologue of BARD1) protein is decreased, and overexpression of either *xBRCA1* or *xBARD1* appears to result in stabilization of the other protein (Joukov, 2001b). McCarthy *et al.* have shown that a similar mutual protein-level control may exist in mice, as *p53*$^{-/-}$, *Bard1*$^{-/-}$ embryos have a decreased amount of Brca1 protein, while *p53*$^{-/-}$, *Brca1*$^{-/-}$ embryos have a decrease in the amount of Bard1 protein (McCarthy, 2003). In the *Xenopus* study, the mRNA levels of the two genes were unaffected, but mRNA levels were not assessed in the mice. To rule out an effect of a change in *Bard1* expression on the phenotype of *gol/gol* cells, *Bard1* expression was assessed by Northern blot, but no change in *Bard1* expression was observed in *gol/gol* ES cells compared to the wildtype control (Figure 6.8b and Table 6.2b).

Overexpression of *p21* has been observed in heterozygous *Brca1* knockout mice, and in mice either homozygous or heterozygous for an exon 11 truncation (Hakem, 1996; Ludwig, 2001). Assessment of *p21* expression

levels in ES cells generated in this study indicated that +/– cells and one of two *gol/gol* cell lines have a slight increase in *p21* expression compared to the wildtype sample (Figure 6.8b and Table 6.2b). Overall, the level of *p21* appears high in these ES cells (irrespective of genotype), countering previous reports of low *p21* expression in ES cells generally (Savatier, 1996). The reason for this is not clear, but could be attributed to a long exposure of the blot.

6.2.8 Brca1 protein is more abundant in cells carrying a *gol* allele

The amount of Brca1 protein present in ES cells of various genotypes was assessed by Western blot analysis. Detection of Brca1 protein was difficult in extracts from wildtype cells, or cells carrying *Brca1⁻* or *c2* alleles, but much easier in those from cells carrying a *gol* allele (Figure 6.10a). A twin Western blot probed with an antibody against α-tubulin demonstrates that roughly equal amounts of protein are loaded for each sample and that degradation of the protein samples does not appear to have occurred (Figure 6.10b). To further provide evidence of roughly even loading, what are presumably non-specific bands at the bottom of the Brca1 blot are also shown (Figure 6.10c). Brca1 protein may be more difficult to detect in wildtype cells due to a combination of low expression and rapid turnover. Blots were detected by ECL.

6.2.9 Alternative reasons for *gol* transcript being more highly expressed and a possible role for suppressors or enhancers of transcription

One striking feature of Figure 6.10 is the apparent difference in protein levels in cells carrying the *gol* allele compared to cells carrying the *Brca1⁻* allele. Expression of the *Brca1⁻* allele does not appear to increase the level of Brca1 protein present in the cell as dramatically as expression of the *gol* allele does. However, this observation re-poses the question of how the *Brca1⁻* and *gol*

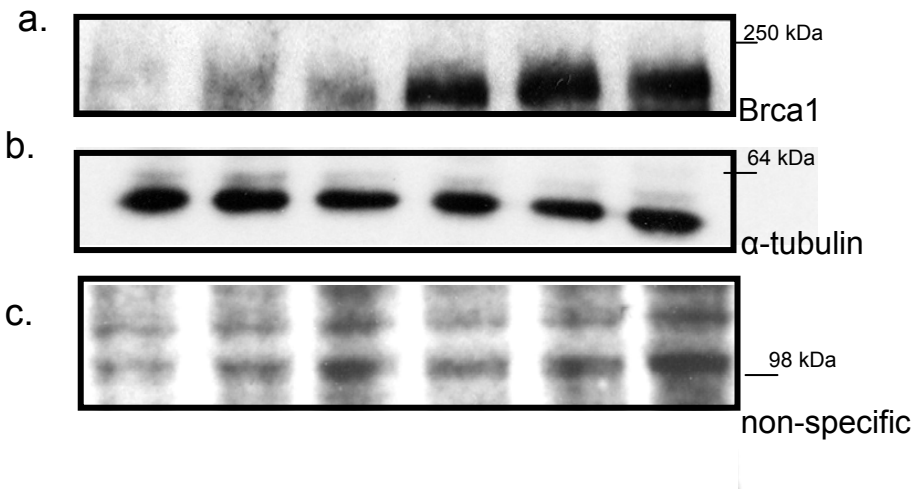


Figure 6.10: An increased amount of Brca1 protein in cells carrying a *gol* allele. a. Western blot of 30 μg total protein isolated from ES cells of the indicated genotypes, detected using the GH118 Brca1 antibody. ECL was used to detect bands. **b.** Duplicate Western blot run with the same samples and at the same time, detected using an α-tubulin antibody. Bands were detected using ECl. c. Secondary bands on from **a,** apparently non-specific, provided as a secondary loading control.
Molecular weights of the SeeBluePlus2 ladder (Invitrogen) are indicated.

alleles differ if the reversed PGK promoter is not involved and the amount of *Brca1* mRNA is similar in *+/–* and *gol/gol* cells.

Mis-expression of *Brca1* in a cell is deleterious; cell cycle arrest and subsequent apoptosis result from overexpression, and loss of Brca1 protein or mRNA expression (as in mouse knockouts or human breast cancers) is also harmful or fatal (Thompson, 1995; Hakem, 1996; Holt, 1996; Liu, 1996; Ludwig, 1997; Sourvinos and Spandidos, 1998). Control of Brca1 at both the protein and mRNA levels is known to occur, allowing differential expression of *Brca1* at different times of development, in response to DNA damage, or at certain stages of the cell cycle (Marquis, 1995; Ruffner and Verma, 1997; Scully, 1997b). As *gol/gol* cells contain a larger amount of Brca1 protein – but not mRNA – than wildtype cells, it seems reasonable to infer that the steady-state level of the different *Brca1* transcripts in ES cells is regulated differently in *+/–* and *+l/gol* cells. Semi-quantitative RT-PCR has shown than the difference in mutant transcript level between *+/–* and *+/gol* cell lines is fairly subtle – the *+/gol* cell line expresses slightly more mutant transcript, and the ratio of wildtype:mutant transcript in this cell line is close to 1:1. However, this assay also suggests that the overall level of *Brca1* is regulated, regardless of genotype, and shows that mutant transcript can be expressed at normal (comparable to wildtype) levels in *gol/gol* or *–/gol* cells. Coupled with the different amount of Brca1 protein detected in *+/–* cells compared to *+/gol* cells, these assays suggest that the proportion of *ΔX.2 Brca1* transcript in relation to the total (or wildtype) amount of *Brca1,* while admittedly subtle, is important. The caveat must be added that a semi-quantitative assay, while more accurate than straight RT-PCR, is not a fully quantitative assay, and could be further supported by one of these assays, such as a TaqMan real-time PCR approach or an RNase protection assay. The advantages to a real-time PCR method include increased accuracy stemming from lack of the need to load samples on gels for analysis (possibly subject to pipetting errors), and the ability to monitor amplification over several cycles, rather than running a set number of cycles before analysis. Further, the mutant and wildtype *Brca1* transcripts could be detected separately by using two assays, which might further increase the accuracy of their quantization. However, semi-

quantitative RT-PCR has been used by a number of labs to determine transcript levels, and when carefully set up and run against TaqMan-type assays, can come very close in accuracy.

What might cause the difference in relative transcriptional amounts? As discussed earlier, the differences between the *Brca1⁻* and *gol* alleles are the selection cassettes (*Hprt* and *Puro*, respectively) and that *gol* lacks ~2 kb more intronic sequence than does *Brca1⁻* (*Brca1⁻* replaces 700 bp of *Brca1* genomic sequence, while *gol* replaces 2.7 kb; Figure 6.1). Therefore, one hypothesis is that either the *Puro* selection cassette in the *gol* allele acts as a transcriptional enhancer, or that an intronic transcriptional repressor normally resides in the intronic region deleted in the *gol* allele. This would help explain why, though the two alleles produce the same transcripts, they are not phenotypically identical. Loss of an intronic suppressor in the *gol* allele is supported by protein data generated from *–/c2* and *–/gol* ES cells. Both the *c2* and *gol* alleles carry the *Puro* cassette, but *c2* has not undergone recombination, so possesses both exon 2 and the surrounding intronic sequence (compare Figure 3.3b and c). Figure 6.10a indicates that *–/c2* cells have protein levels comparable to wildtype cells, while *–/gol* cells have a greater amount of protein than wildtype cells. Further, Figure 6.9 indicates that *–/c2* cells have an amount of wildtype *Brca1* transcript similar to that of *+/–* cells, suggesting that the *c2* allele is still being regulated as a wildtype-like allele. This suggests that deletion of the intronic region may be linked to increased protein levels, although it may also simply reflect the presence of exon 2, and, presumably, normal *Brca1* production. There is the alternative explanation that some type of *trans* effect could be occurring in which the *gol* transcript interferes with the turnover or transcription of the wildtype transcript.

Unfortunately, a reliable prediction program for modifiers of transcription does not yet exist to help confirm this hypothesis. However, intronic modifiers of transcription, both enhancers and repressors, have been widely reported in the literature. For example, Brinster *et al.* generated transgenic mice which expressed the rat growth hormone gene, either with or without introns, under the control of a liver or pancreas-specific promoter. They found that

expression of the transgene was much higher when the introns were present (Brinster, 1988). Additionally, effective expression of both the mouse and human *Hprt* genes depends on the presence of certain introns (Reid, 1990; Magin, 1992).

Regardless of genotype, the level of *Brca1* mRNA is fairly consistent in all cell lines tested in this study. This suggests that the protein translated from the *Brca1 ΔX.2* transcript may be more stable than is wildtype Brca1. This could help explain the larger amount of Brca1 protein detected in cells carrying the *gol* allele compared to the other cell lines (Figure 6.10). Figure 6.11 shows a model which attempts to integrate the data presented in this chapter.

6.2.10 Brca1^{gol} and Bard1 have decreased interaction in a pull-down assay

As described in section 6.2.1, translation of Brca1^{gol} from the *Brca1 ΔX.2* transcript likely initiates at one of the AUG codons in exon 5 or 6. Regardless of which AUG is used, the resulting protein will lack part of the N-terminal RING domain. In previous analyses by other groups, the interaction of BRCA1 and BARD1 was abrogated by certain point mutations in the RING domain of BRCA1; the effect of a larger deletion is likely to be equally detrimental (Wu, 1996; Morris, 2002). To investigate if Bard1 can interact with Brca1^{gol}, glutathione-S-transferase (GST)–Brca1 and myc-Bard1 fusion proteins were generated and co-expressed in mammalian 293T cells. Full-length *Bard1* cDNA was cloned into an N-terminal c-myc–tag vector, while pieces of *Brca1* (from exons 2, 3, 5, or 6 to exon 10) were cloned into an N-terminal GST-fusion vector (Figure 6.12). These four Brca1 fusions mimic, respectively, wildtype Brca1, an exon 2 deletion, Brca1 initiating at the exon 5 AUG, or Brca1 initiating at the first AUG in exon 6 (the latter two begin at the codon following the AUG).

Immunoprecipitation of GST-Brca1 fusions was followed by Western blot analysis using a c-myc antibody to detect the Bard1 fusion, and vice versa. Figure 6.13 demonstrates that, unfortunately, the c-myc antibody cross-reacts

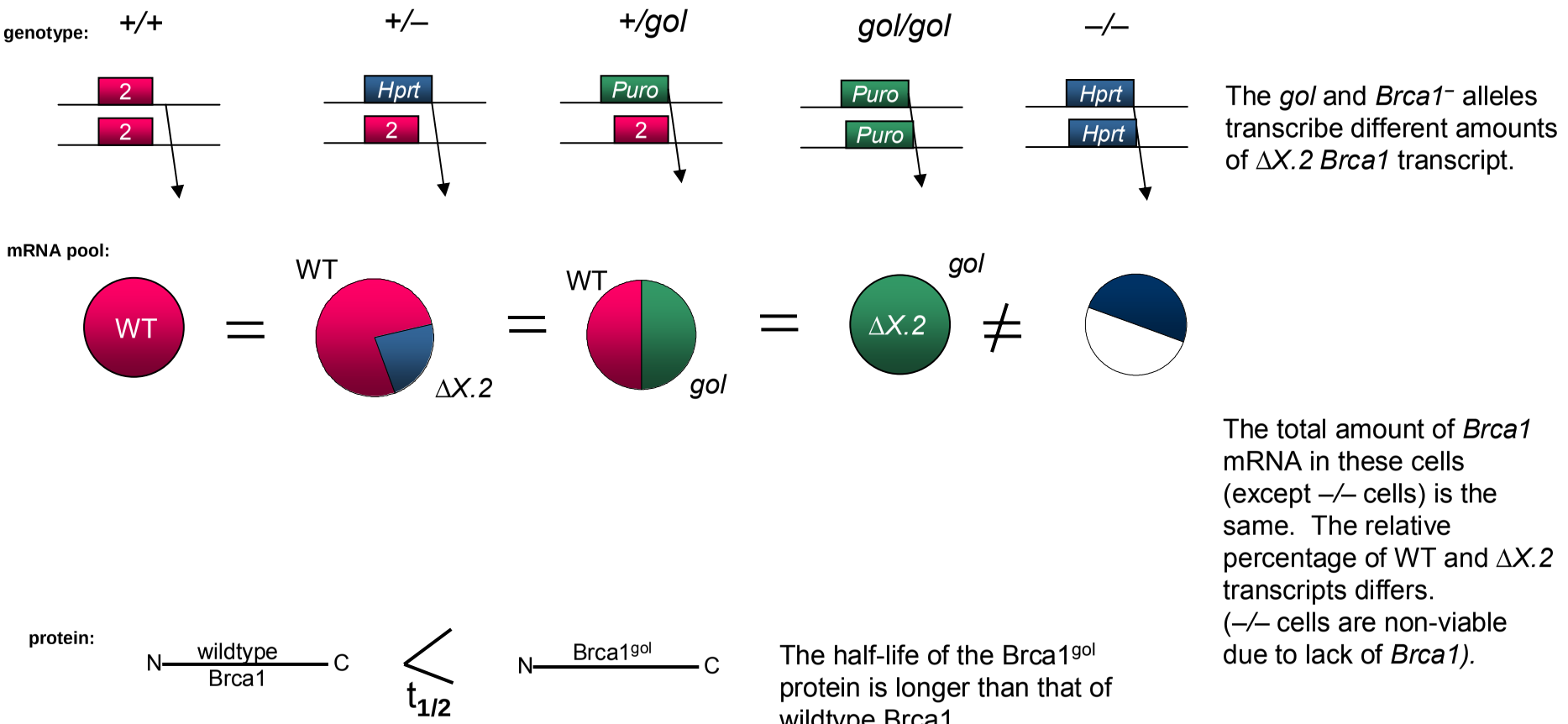


Figure 6.11: Model of differential expression of the *Brca1⁻* and *gol* transcripts. Both the *Brca1⁻* and *gol* alleles produce the *Brca1* Δ*X.2* transcript, but this model suggests that the Δ*X.2 (gol)* product is more abundantly transcribed by the *gol* allele. Although the overall level of *Brca1* mRNA is equivalent in all viable ES cells, a larger proportion of the overall mRNA in *+/gol* cells is comprised of the *Brca1* Δ*X.2* transcript. –/– cells are not viable because they do not produce sufficient *Brca1* transcript. Furthermore, the Brca1 ΔX.2 protein is predicted to be more stable than wildtype Brca1.

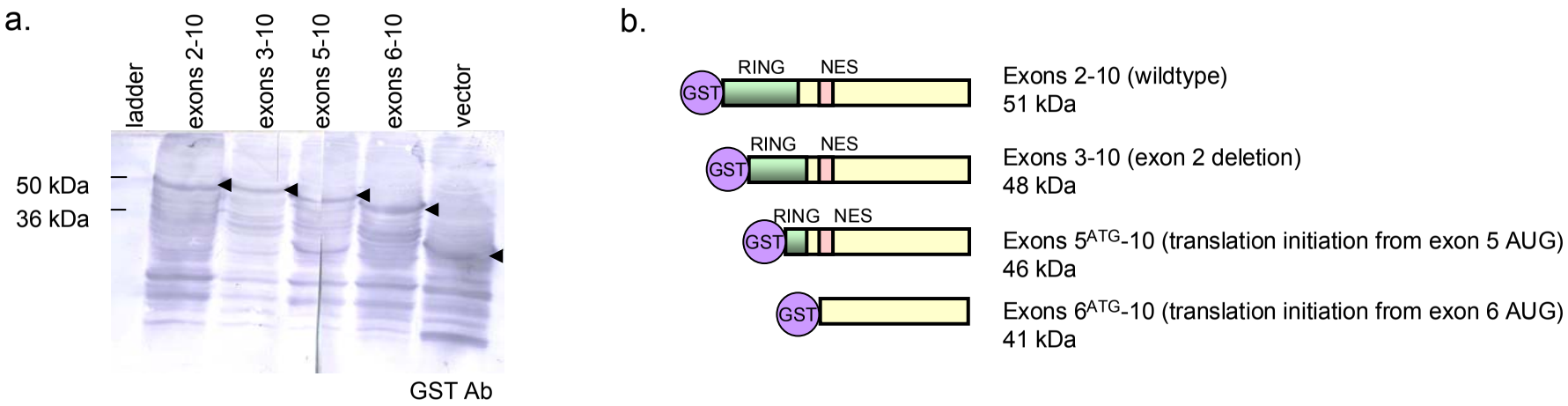


Figure 6.12: GST-Brca1 and myc-Bard1 fusion proteins. a. Western blot analysis of N-terminal GST-Brca1 fragment (exons 2, 3, 5, or 6 to exon 10) fusion proteins; arrows indicate correct products. 30 μg of total protein extract from 293T cells transiently transfected with fusion protein vector or empty vector was loaded per lane. Expected sizes are 51, 48, 46, or 41 kDa, respectively (empty vector, 27 kDa). **b.** Schematic of the four GST-Brca1 fusion proteins and what they mimic. **c.** Western blot analysis of N-terminal myc-tagged, full-length Bard1 (arrow). 30 μg of total protein extract from 293T cells transiently transfected with the myc-Bard1 fusion protein vector was loaded per lane. c-myc antibody was used for detection. Expected size of the fusion protein is ~90 kDa. Approximate molecular weights of the SeeBluePlus2 ladder (Invitrogen) are indicated. Ab=primary antibody used for detection, GST=glutathione S-transferase, NES= nuclear export signal.

with the GST-Brca1 fusions. All four GST-Brca1 fusions are detected by the c-myc antibody following a GST immunoprecipitation (Figure 6.13a, lanes 2-5), and are additionally immunoprecipitated by the c-myc antibody (Figure 6.13b, lanes 3-5). The c-myc antibody does not appear to immunoprecipitate the empty GST fusion vector (Figure 6.13b, lane 2), and the cross-reaction was not abrogated by more stringent washing of the antibody-antigen-bead complex following the immunoprecipitation reaction (done on samples in Figure 6.13b).

Fortunately, myc-Bard1 alone was neither detected nor immunoprecipitated by the GST antibody (Figure 6.13b, lane 6 and Figure 6.13c), so a GST immunoprecipitation could still be used to assess the interaction of myc-Bard1 and GST-Brca1. Figure 6.13a indicates that the GST-Brca1(exons 2-10) fusion protein, which mimics wildtype Brca1, appears to bind strongly to myc-Bard1. A decreased amount of binding is demonstrated by the exons 5-10 (lane 12) construct, and a very faint band can be seen in the exons 6-10 (lane 13) construct. Detection of myc-Bard1 with a c-myc antibody following a c-myc-immunoprecipitation (Figure 6.13a, lanes 6-9) provides a control for the amount of myc-Bard1 which should be detected following immunoprecipitation. This amount is very similar to the amount co-immunoprecipitated with GST-Brca1(exons 2-10) in lane 10. The failure to see any binding in the exons 3-10 lane, which should theoretically resemble the other two mutations, may be because of improper folding of the protein. However, it should be noted that no loading control is used on these blots, leaving interpretation of relative amounts of protein open to some question.

This experiment indicates that Bard1 and Brca1^{gol} are, depending on where the translational start site is, likely to interact at a decreased level. A decrease (vs. abrogation) in binding is somewhat surprising considering the structural data (Figure 6.2) and previous experiments describing the effect of point mutations on the interaction (Wu, 1996; Brzovic, 2001b). However, the reduced amount of binding observed may be due in part to the assay, in which large amounts of protein are transiently expressed in a cell line before the pulldown is performed. It would be worthwhile to attempt to co-

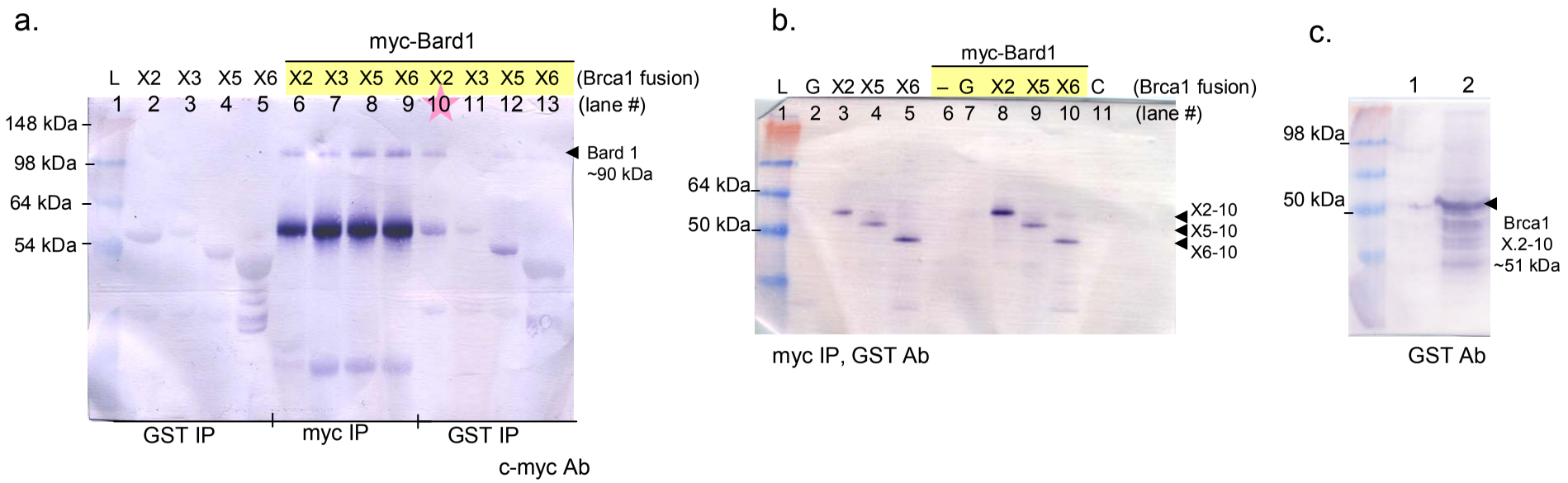


Figure 6.13: Co-immunoprecipitation of GST-Brca1 and myc-Bard1 fusion proteins. a. c-myc-probed Western blot of GST or c-myc immunoprecipitations (IPs) from cells expressing the indicated fusion protein vectors. Lanes 2-5 show that the c-myc antibody (Ab) immunoprecipitates the GST-Brca1 fusion proteins non-specifically. Lanes 6-9 indicate that the myc-Bard1 protein (90 kDa band, arrow) is immunoprecipitated and recognized by the c-myc antibody; the other two bands are the c-myc Ab heavy and light chains, detected by the secondary Ab. Lanes 10-13 indicate that only GST-Brca1(exons 2-10) successfully co-IPs the myc-Bard1 fusion. **b.** GST-probed Western blot of c-myc IPs from cells expressing the indicated fusion proteins. Lanes 3-5 show that the c-myc antibody recognizes the GST-Brca1 fusion proteins (even after more stringent washing), but not the empty GST vector (lane 2). Lane 6 indicates that myc-Bard1 is not immunoprecipitated by the GST antibody. Lane 11 contains extract from non-transfected cells. **c.** GST-probed Western blot. 30 μg of protein from cells expressing only the myc-Bard1 fusion (lane 1) or GST-Brca1(exons 2-10) fusion protein (lane 2, arrow), indicating that myc-Bard1 is not recognized by the GST antibody. Approximate molecular weights of the SeeBluePlus2 ladder (Invitrogen) are indicated. IP=immunoprecipitation, Ab=primary antibody, X=exon, L=ladder, G=GST empty vector.

imunoprecipitate Brca1^{gol} from *gol/gol* ES cells using a Bard1 antibody to confirm that the two can interact *in vivo* when expressed and regulated at normal levels.

Previous *in vitro* studies using transiently expressed BARD1 and BRCA1 have indicated that when the RING domain of BRCA1 is mutated or lost, BRCA1 is found mostly in the cytoplasm, presumably due to the presence of both NLSs and the NES (Fabbro, 2002). The Brca1^{gol} protein localizes to the nucleus and cytoplasm, and its localization is very similar to that of wildtype Brca1. This suggests that, in addition to the loss of the RING domain, the NES may be mutated or lost in Brca1^{gol}, as would be the case if translation initiated from the second downstream AUG. *In vitro* data has indicated that the NLSs coded by exon 11 are sufficient to direct BRCA1 to the nucleus in the absence of the NES (Rodriguez and Henderson, 2000). Although it appears that Bard1 may be able to interact with Brca1^{gol}, it is unknown at present if the lowered amount would be sufficient for all Brca1^{gol} to be properly chaperoned, so the loss of the NES may well be important.

6.3 DISCUSSION

Initially, the structure and viability of the *gol* allele led to speculation that the PGK promoter from the *Puro* selection cassette functioned as a secondary promoter to drive expression of *Brca1* starting from a downstream, in-frame AUG codon. Such downstream AUG codons do exist at the 5' end of Brca1, in exons 5 and 6. However, while the PGK promoter does appear to be able to function bidirectionally in ES cells (Table 6.1), no evidence for its involvement in *Brca1* expression has been observed in the experiments described in this chapter. Reports of aberrant transcripts driven by a reversed PGK promoter are not particularly prevalent in the literature, but in the two cases when the resulting aberrant transcript was sequenced, part of the reversed promoter itself was present in the transcript (Abeliovich, 1992; Scacheri, 2001). No such sequence was observed in transcripts of *gol/gol* ES cells as assessed by Northern blot, RT-PCR, or 5' RACE (Figures 6.5, 6.8, and data not shown).

6.3.1 Transcriptional control of the *gol* and *Brca1*$^{-}$ alleles

While 5' RACE analysis indicated that *gol/gol* cells express both the *Brca1 ΔX.2* isoform, which splices from exon 1 to exon 3 (Figure 3.9) and a smaller novel transcript, the *Brca1 ΔX.2* product is also produced by the *Brca1*$^{-}$ allele, and the smaller transcript was detected in both *+/–* and wildtype samples (Figure 6.5). Northern blot analysis similarly failed to detect a secondary transcript which might have originated outside the region analyzed by 5' RACE (Figure 6.8a). These analyses suggested that the *gol* allele does not produce a unique transcript, at least not at a level detectable by these assays. It is doubtful that Brca1^{gol} is a dominant negative form of Brca1, as *+/gol* and *gol/gol* cells would then be expected to have very similar phenotypes. While these two cell lines both have increased levels of Brca1 protein, *gol/gol* cells are hypersensitive to MMC and γ-irradiation, while *+/gol* cells are not (Figures 6.10, 5.4, and 5.5).

Northern blot analysis and semi-quantitative RT-PCR indicated that *Brca1* mRNA does not appear to be overexpressed from the *gol* allele, as might have been expected (Figure 6.8a, Figure 6.9). In fact, the overall level of *Brca1* mRNA was very similar amongst all the genotypes tested, both by Northern blot, and by semi-quantitative RT-PCR assay (Figure 6.8a and Figure 6.9). Only one group has included *Brca1* expression data when describing their *Brca1* ES cell lines, but in that case, expression of *Brca1* did not appear to differ greatly between wildtype and heterozygous ES cells (Gowen, 1996). This may be a result of regulation of *Brca1* mRNA levels in the cell – although certainly loss of one copy of *Brca1* might lead to loss of up to half the normal gene product. Data from many studies has demonstrated that overexpression or loss of *Brca1* is deleterious (Thompson, 1995; Hakem, 1996; Holt, 1996; Liu, 1996; Ludwig, 1997), and that Brca1 is regulated at both the mRNA and protein level during development, pregnancy, lactation, the cell cycle, and following DNA damage (Marquis, 1995; Ruffner and Verma, 1997; Scully, 1997b). The similarity of *Brca1* expression levels

observed in this study amongst ES cells of different genotypes may be a consequence of such regulation.

The inviability of *–/–* ES cells and mice, but viability of *gol/gol* ES cells suggests that the *Brca1⁻* allele may underexpress Brca1 transcript or protein. In Figure 6.9, it is shown that in *gol/gol* or *gol/–* ES cells, *Brca1* appears to be expressed at levels similar to those detected in wildtype cells. Although the overall *Brca1* mRNA level is also similar in *+/–* and *+/gol* ES cells, the proportion of transcript from the alleles differs (1:3.1 compared to 1:1.2). The absolute amount of mutant product in the two cell lines is only subtly different; the relationship of the wildtype and mutant products is significantly different. This may indicate that some type of *trans* effect regulates the level of wildtype and mutant transcripts in *+/gol* or *+/–* cells (which differs between the *gol* and *Brca1⁻* alleles), or it may reflect a difference in the transcriptional regulation of the *gol* and *Brca1⁻* transcripts. This could result from the differences between the *Brca1⁻* and *gol* alleles at the primary sequence level: the selection cassettes and the amount of intronic region deleted. The *gol* allele may have lost an intronic transcriptional repressor, or the *Puro* selection cassette may act as a transcriptional enhancer. Deletion of a transcriptional repressor seems the more likely explanation, as the *c2* allele also carries the *Puro* selection cassette (but retains the intronic regions) and *–/c2* ES cells, unlike *–/gol* ES cells, do not have more Brca1 protein than wildtype cells (Figure 6.10a), and are not hypersensitive to γ-irradiation (Figure 5.4). A small schematic which sums up this model of relative amounts of transcription from the *Brca1⁻* and *gol* alleles is presented in Figure 6.14.

Many examples of intronic enhancers or suppressors have been reported in the literature (Reid, 1990; Magin, 1992; Ash, 1993; Jonsson, 1994; Oskouian, 1997; Scohy, 2000). Such modifiers have often been identified during studies of promoter regions, and as such are frequently located in the first intron following the translational start site. However, enhancers are found in many locations – in the 5' and 3' UTRs, and within genes; tissue-specific expression of the murine gene *Pax6* in the iris and amacrine cells appears to be regulated by an enhancer in intron 4 (Xu, 1999a). Brinster *et al.* have

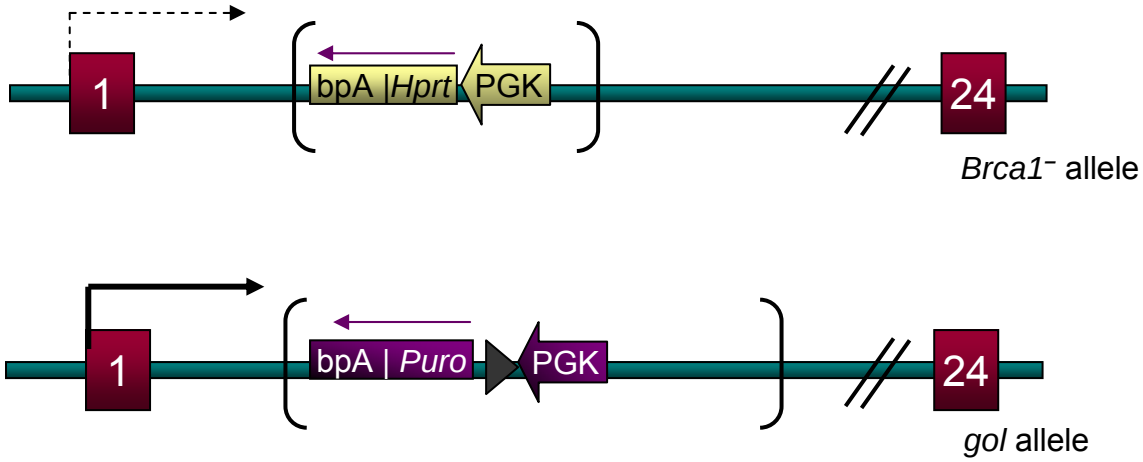


Figure 6.14: The *gol* and *Brca1*⁻ alleles of *Brca1*, model of transcription. Model of transcription of the *gol* and *Brca1*⁻ alleles of *Brca1.* The amount of genomic DNA deleted in the two alleles is indicated by black brackets. Transcription, as indicated by the arrows, is more robust from the *gol* allele than from the *Brca1*⁻ allele. Different amounts of transcription may be due to loss of a transcriptional suppressor in the area deleted in *gol* but not in *Brca1*⁻, or because the *Puro* selection cassette serves as a transcriptional enhancer.

additionally shown that the expression of intron-containing transgenes in mice is higher than expression of the same transgene without the introns (Brinster, 1988). Additionally, effective expression of both the mouse and human *Hprt* genes depends on the presence of certain introns (Reid, 1990; Magin, 1992). A repressor sequence has been identified in intron 1 of human *BRCA1*; this sequence will repress transcription of a reporter gene to which it is linked. However, a similar repressor sequence could not be identified in introns 1 or 2 of murine *Brca1* (Suen and Goss, 2001). A mouse-human intron 2 alignment did identify two regions of homology, but both are located downstream of the *gol* deletion.

Although the difference in mRNA levels in the *+/–* and *+/gol* cell lines is subtle, it does appear to have an effect on the cells, and the amount of protein in the two cell types does appear to differ. Given the small differences seen in the semi-quantitative RT-PCR assay, it is desirable to move to a more quantitative method, such as real-time PCR (as discussed earlier). The semi-quantitative RT-PCR assay relied on running aliquots of reactions on agarose gels followed by analysis of the resulting digital images. Real-time PCR reduces pipetting/analysis errors, and also is able to measure the amount of product per cycle instead of using a "snapshot" of the reaction at one or two points, and should reduce the sample-to-sample variability. Furthermore, a TaqMan assay can be defined that measures the mutant and the wildtype products separately, which may have a bearing on the analysis. Alternatively, an RNase protection assay involving exons 1-3 could be used to assess the relative amounts of transcript produced from the wildtype and mutant alleles in *+/gol* and *+/–* cells. The results of either assay should help generate a more accurate model of transcription of *Brca1* in cells carrying the *gol* allele.

6.3.2 Regulation of the level of Brca1^{gol} protein

One consequence of expression of *Brca1* from the *gol* allele appears to be a greater amount of Brca1 protein. As *gol/gol* cells do not overexpress *Brca1* mRNA, the greater level of protein may indicate that the Brca1^{gol} protein has a longer half life or is translated more efficiently than wildtype Brca1. Increased

stability of Brca1^{gol} was discussed in Chapter 5 as a possible explanation for the presence of aggregates of Brca1 protein in *gol/gol* cells both before and after γ-irradiation (although the presence of aggregates may also be explained by an antibody or cell-based artifact).

While the hypothesis that the region deleted in Brca1^{gol} contains an instability sequence is logical, prediction programs suggest that the putative Brca1^{gol} protein is not more stable than wildtype Brca1, and that the N-terminus of Brca1 contains no PEST domains (protein domains enriched in proline (P), glutamic acid (E), serine (S), and threonine (T), which may serve as proteolytic signals (Rechsteiner and Rogers, 1996; Bioinformatics1, 2003; Rogers, 2003)). However, prediction programs are limited by available information, and the presence of other instability sequences was not investigated.

An alternative explanation for the increased amount of Brca1 protein in *gol/gol* cells may involve the decrease in the Brca1-Bard1 interaction in these cells. In human cells, the BRCA1/BARD1 heterodimer is known to undergo autoubiquitination, a modification postulated to stabilize both components of the heterodimer, suggesting that free BRCA1 or BARD1 is less stable than the heterodimer (Chen, 2002; Mallery, 2002). Additionally, mutual control of Brca1 and Bard1 protein levels has been suggested by experiments involving *Bard1* and *Brca1* knockout mice or *Xenopus* embryos following antisense-mediated depletion of xBRCA1 or xBARD1. These experiments indicated that loss or depletion of one protein results in what appears to be a reciprocal decrease in the amount of the other protein, while overexpression of human or *Xenopus BARD1* or *BRCA1* in cultured human 293T cells results in increased stability of the other protein (Joukov, 2001a; Joukov, 2001b; McCarthy, 2003). This appears to contrast with data from *gol/gol* cells, in which the mutant Brca1^{gol} protein is more abundant or stable than wildtype Brca1 (despite being less prone to heterodimerization with Bard1), but the two observations may be in agreement. Mutual protein-level control may involve the less-stable free form of a more abundant partner stabilizing the second protein, in an attempt to gain a binding partner. Therefore, in the absence of

Brca1, Bard1 would appear to be less abundant than normal, because it is present only in its less-stable free form. In *gol/gol* cells, where the Brca1^{gol} protein is generated but Bard1 interaction is decreased, free Bard1 may stabilize Brca1^{gol} to try and find a binding partner. This could explain the increased amount of Brca1 protein detected in cells carrying the *gol* allele. More experimentation will be required to resolve this issue, and the use of *gol/gol* cells may provide a good tool for further investigation into the control of Brca1 protein levels in the cell.

6.3.3 Bard1 and Brca1

The *gol* allele may prove to be a useful model for studying the Brca1-independent functions of Bard1. Bard1 appears to be necessary for a DNA damage-induced pause in transcriptional processing (Kleiman and Manley, 1999; Kleiman and Manley, 2001), and may itself be a tumour suppressor, though little evidence to support this hypothesis has been reported to date (reviewed in Irminger-Finger and Leung, 2002). Although it has been suggested that not all cellular Bard1 interacts with Brca1 (Chiba and Parvin, 2002), the pivotal role of Bard1 as a Brca1 binding partner and nuclear chaperone (and, now, the role of Brca1 as a Bard1 chaperone (Rodriguez, 2004)) has so far precluded study of its other roles. For instance, the *Bard1* knockout mouse is phenotypically identical both to a *Brca1* knockout mouse and to *Brca1/Bard1* double knockout mice (McCarthy, 2003). The availability of a form of Brca1 which does not interact at normal levels with Bard1 but does localize to the nucleus may help to uncover the other functions of Bard1, as well as to reveal the importance of other roles of the Brca1/Bard1 heterodimer, such as its E3 ubiquitin ligase activity. To the knowledge of this author, the *gol* allele is the only allele of *Brca1* which would allow this topic to be investigated without introducing protein into the cell by artificial expression. Additionally, it would be interesting to pair the *gol* allele with the published *Bard1* knockout allele.

In summary, the *Brca1*$^{-}$ and *gol* alleles, though similar, have very different effects on the cells which carry them. The *gol* allele may serve as a very

useful model for uncovering both the Brca1–independent functions of Bard1 and for investigation of the roles of the Brca1/Bard1 heterodimer.

CHAPTER SEVEN:

DISCUSSION
AND FUTURE PLANS

7.1 INTRODUCTION

The study of *BRCA1* and its functions has now spanned over a decade. An enormous amount of work has been done to describe the functions of this gene and the consequences of *BRCA1* mutations, both in humans and in model organisms. Despite this, a number of questions still remain: what is the mechanism by which loss of BRCA1 causes breast cancer? Which of the many interactions are biologically relevant, and how do they contribute to tumourigenesis and the normal functions of BRCA1? What are the normal roles of BRCA1 in the cell, and which of these functions are conserved between species?

In the preceding chapters, the generation and characterization of several novel mutant alleles of murine *Brca1* have been described. The *Brca1*$^{-}$ allele generated in this study (*Brca1*^{Brdm1}) has the hallmarks of previously-defined null knockout alleles: double-targeted ES cells could not be generated, homozygous mutant embryos were not viable, and heterozygous mutant mice did not have an increased tendency to tumourigenesis, even on an *Blm*$^{-/-}$ background. This allele was generated for two purposes: as part of a conditional ES cell line, and to be tested in conjunction with a *Blm* mutation in tumourigenesis studies. However, most of the work described in previous chapters has focused on the novel allele *Brca1*^{Brdm2} or *gol*, which generates an N-terminal truncated Brca1 protein predicted to be missing the majority of the highly-conserved RING domain. The viability of this allele in homozygous ES cells was unexpected, based on the similarity between it and the *Brca1*$^{-}$ allele, but, as described in Chapter 6, it seems likely that these two alleles are regulated differently. This chapter includes some general discussion points which follow on from the discussions included in previous chapters, and describes several future studies which might be performed using this novel allele.

7.2 THE *gol* ALLELE AND HUMAN *BRCA1* MUTATIONS

The *gol* allele is unique among mouse mutant *Brca1* alleles in that it generates an N-terminal truncation of the Brca1 protein, resulting in a deletion of the highly-conserved Zn-finger RING domain of BRCA1. This domain has been conserved in BRCA1 homologues from plants to *C. elegans* to mammals (see Figure 1.11), and is very highly conserved between mouse and human (see Figure 1.3). To date, no report of a functional N-terminal truncated human BRCA1 protein has been published, although one group has reported a mutation in the "initiation" codon of pseudo-exon 2 of *BRCA1* (in the 5' UTR of *BRCA1*), and *in vitro* studies have indicated that if this mutation occurred in the *BRCA1* gene itself, transcription would re-initiate at a downstream AUG (Signori, 2001). Four different mutations of the AUG initiation codon in exon 2 have been reported in the Breast Cancer Information Core (BIC) database, and it is possible that re-initiation may occur in these alleles (BIC, 2003). The human *BRCA1* transcript possesses fewer alternative start codons than the mouse, but there is a second AUG in exon 2 which occurs in a good consensus Kozak setting (the second, in exon 5, is not so well-placed). According to the BIC, mutations in the RING domain/exon 2 account for ~24%/18% of reported mutations in *BRCA1*. Whether any of these mutations result in re-initiation is unknown (BIC, 2003).

7.3 ALTERNATIVE ALLELES OF *Brca1*

Brca1^{-} and *gol* join a growing list of mutant alleles of murine *Brca1*. This list has expanded to include several conditional mutations, a C-terminal truncation allele, and alleles with partial function. The latter group includes alleles such as the *Brca1* $\Delta X.11$ allele which mimics a natural splice isoform (found in both humans and the mouse) lacking the whole of exon 11. One group has generated one ΔX.11 double-targeted ES cell line and a small number of *Brca1*$^{\Delta X.11/\Delta X.11}$*, p53*$^{-/-}$ mice (Table 1.3 #5) (Gowen, 1996; Cressman, 1999a; Cressman, 1999b); another has shown that their *Brca1* $\Delta X.11$ allele is viable on a *p53*$^{+/-}$ or *p53*$^{-/-}$ background, although the mice tend

to develop cancers, including thymic lymphomas, especially on the *p53*$^{-/-}$ background (Table 1.3 #7) (Xu, 2001b; Bachelier, 2003).

Ludwig *et al.* have generated a very interesting alternative allele of *Brca1*; this allele mimics the results of a human cancer-related nonsense mutation within exon 11. This allele generates a C-terminal truncation product lacking nearly half the protein, yet homozygous mutant mice are viable (depending in part on strain background). Male homozygotes were infertile, but homozygotes were generally healthy, although prone to tumours (86% of animals developed tumours of some type after a mean latency of 1.4 years (Ludwig, 2001)). Mammary tumours were observed in some mice, but the overall tumour spectrum encompassed lymphomas, sarcomas, and adenomas in various tissues. The long tumour latency suggested that the mutated Brca1 protein was not the only factor responsible for tumourigenesis, and further study of some tumours indicated that the expression of several other gene products was altered (interestingly, the amount of *p53* gene product was altered in only 3 of 10 mammary tumours tested). They also observed, like Bachelier *et al*., that animals on a *p53*$^{-/-}$ background developed thymic lymphomas more rapidly than did *p53*$^{-/-}$ control animals (Ludwig, 2001; Bachelier, 2003).

These models indicated that mice homozygous for a *Brca1* mutation can shorten the tumour latency of *p53*$^{-/-}$ mice, supporting the role of Brca1 as a caretaker tumour- suppressor in mice (Ludwig, 2001; Bachelier, 2003; Jonkers and Berns, 2003). *Brca1* may have similar roles in the cell as does human *BRCA1*, but the fact remains that heterozygous murine carriers of a *Brca1* mutation do not have an increased predisposition to tumourigenesis compared to wildtype mice, in contrast to human mutation carriers. In numerous studies, *Brca1*$^{+/-}$ mice on a *p53* mutant background, an *Apc*$^{+/min}$ background, and (in this work), on a *Blm*$^{-/-}$ background, do not speed the time-to-tumour compared to mice without a *Brca1* mutation (Cressman, 1999b; Hohenstein, 2001). This was also the experience of Jonkers *et al.* who showed, using mice co-conditional for both *Brca1* and *p53*, that tumours formed more rapidly in *Brca1*$^{-/-}$, *p53*$^{-/-}$ mice than in *Brca1*$^{+/-}$, *p53*$^{-/-}$ mice, but that all tumours which did form had lost both copies of *p53* (Jonkers and

Berns, 2003). These experiments suggest that the *Brca1*-related tumour profile in mice may differ from the spectrum developing in human heterozygous *BRCA1* mutation carriers (Lane, 1995; Marquis, 1995; Scully, 1997c; Chen, 1998).

7.4 DIFFERING TUMOUR SPECTRA AND A RANGE OF PHENOTYPES: TUMOUR SUPPRESSOR MODEL SYSTEMS

Neither the range of phenotypes observed in mice carrying different alleles nor the difference between mouse and human tumour spectra nor the are rare events amongst models of human cancer-related genes. As was mentioned in Chapter 4, mouse *Brca2* mutant alleles have a similar range of viability, including null alleles which are embryonic lethal (Ludwig, 1997; Sharan, 1997) and a range of alternative alleles. Mice homozygous for some of these alleles succumb early in life to tumours, generally thymic lymphomas (Connor, 1997; Friedman, 1998). An allele which deletes exon 27 results in viable homozygous mice and cells which are hypersensitive to DNA damaging agents (mice are prone to tumourigenesis at an earlier age than heterozygotes or wildtype animals (Morimatsu, 1998; Donoho, 2003)). The three published mutant alleles of *Blm* also differ from one another; the one used in this study generates viable homozygous mutant mice with an increased tumour predisposition that will accelerate tumourigenesis in $Apc^{+/min}$ mice (Luo, 2000). However, mice homozygous for the other two alleles are not viable, and these alleles appear to be haploinsufficient, as a heterozygous mutant background will accelerate tumourigenesis in $Apc^{+/min}$ mice (Chester, 1998; Goss, 2002).

Neurofibromatosis type 2 (NF2) is a familial dominant disorder characterized in humans by schwannomas and meningiomas caused by a germline mutation in one copy of *NF2*. *NF2* mutations are also found in sporadic schwannomas. While $Nf2^{+/-}$ mice are cancer-prone, they exhibit osteosarcomas and hepatocellular carcinomas at an advanced age, and null mutants die in early embryogenesis, which is not an accurate model of the human condition (McClatchey, 1997; McClatchey, 1998). Mice carrying

mutant *Rb1* alleles are cancer-prone, but they do not accurately mimic the human phenotype (including retinoblastoma, a childhood malignancy of the retina, as well as osteosarcomas, prostate, and breast cancers (reviewed in Zheng and Lee, 2001). *Rb1*$^{+/-}$ mice develop pituitary gland tumours between 6 and 8 months of age, and *Rb1*$^{-/-}$ mice die in late embryogenesis, although the developing retina appears normal (Clarke, 1992; Jacks, 1992; Lee, 1992). In both cases, better models of the human condition were generated by using either conditional alleles driven by tissue-specific Cre transgenes, or secondary mutations (Lee, 1996; Robanus-Maandag, 1998; Giovannini, 2000). This is not unlike the use of *WAP*-, MMTV-, or *K14*- Cre transgenes in conjunction with *Brca1* conditional mice to try and restrict expression to the mammary gland (Xu, 1999b; Jonkers and Berns, 2003).

The embryonic lethality of homozygous mutant *Brca1* mice and the predisposition to cancers in heterozygous *BRCA1* carriers has often been described as paradoxical, as these two outcomes describe cell death and uncontrolled cell growth, respectively. However, these two outcomes are likely to be consistent with *BRCA1* being a tumour-suppressor gene expressed in growing and differentiating cells. In the cells of an adult human carrier of a *BRCA1* mutation, loss or mutation of the second allele is likely to lead to genomic instability, which eventually leads to cancer via additional mutations acquired in the cells. This is exactly what is likely to occur in embryonic tissues of *Brca1*-mutant homozygotes, too: loss of *Brca1* leads to additional mutations, which eventually leads to enough genetic disorder that the cell can no longer function. However, unlike the hypothetical adult cell, the homozygous mutant cells of the embryo are rapidly undergoing a huge amount of growth and differentiation, and the burden of mutations is likely to become overwhelming more quickly. Additionally, *Brca1* is normally expressed in the developing embryo, which suggests that any effects of its loss would likely be observed more quickly than in an adult cell where it may not be expressed (Marquis, 1995). An increased burden of mutations in *Brca1*-mutant embryos is supported by the studies of Shen *et al.* who showed that chromosomal instability is increased in *Brca1*$^{\Delta X.11/\Delta X.11}$ embryos compared to wildtype embryos (Table 1.3 #6). The addition of a second mutation (*p53*

deficiency) resulted in a greater amount of genomic rearrangement (Shen, 1998).

Based on these previous studies, the *gol* allele may serve as a useful model for *BRCA1*-related tumourigenesis, but may be more advantageous when used in studies of the functions of *Brca1* in the cell (which indirectly apply to tumourigenesis). The original aim of this project was to investigate the functions of *Brca1* in the cell, with a view to understanding the molecular causes behind its role in tumourigenesis. The *gol/gol* cells provide a tool for investigating the functions of Brca1, both in well-studied areas such as the response to γ-irradiation, nuclear focus formation, and phosphorylation, and in others such as nuclear import and export, RING-domain interactions and their consequences, degradation of the Brca1 protein, and possible targets of the Brca1-Bard1 E3 ligase.

7.5 THE FUNCTIONS OF BARD1

Bard1 was isolated on the basis of its interaction with Brca1, and the majority of subsequent studies involving this protein have focused on its functions in tandem with Brca1. A few studies investigating Bard1 as a possible tumour-suppressor gene have unanimously concluded that Bard1 is rarely, if at all, involved in mammary tumourigenesis (Thai, 1998; Yoshikawa, 2000; Ishitobi, 2003). However, there have been some glimpses into the functions of Bard1, including possible roles in cell cycle control and the response to DNA damage.

Antisense-mediated depletion of *Bard1* in cultured cells has indicated that cells with decreased expression of *Bard1* tend to have a prolonged cell cycle and a higher amount of aneuploidy or polyploidy (Irminger-Finger, 1998). *Bard1* also appears to be transcriptionally upregulated following genotoxic stress (UV treatment), and may be upregulated or induced during apoptosis (Irminger-Finger, 2001). A second group has shown that UV exposure or a DNA replication block induced by HU treatment results in a temporary block in 3’ cleavage of pre-mRNAs. BARD1 is likely to be necessary for this damage-

induced block, as it does not occur if BARD1 is mutated (Kleiman and Manley, 2001). Overexpression of BARD1 also appears to induce apoptosis and increase the amount of p53 in the cell – similar to what has been observed in cells overexpressing BRCA1 (Irminger-Finger, 2001).

BARD1 may also play a role in homologous recombination repair (HRR); when a dominant-negative truncated version of BARD1 (BARD1 can interact with BRCA1 but is missing its C-terminus), is transfected into *Brca1*$^{+/+}$ or *Brca1*$^{\Delta X.11/\Delta X.11}$ ES cells (Table 1.3 #5), an I-SceI repair assay indicates that both cell lines have a lower efficiency of HRR than the parental cell line (Westermark, 2003). *gol/gol* cells could be used to determine if the interaction of Bard1 and Brca1 was necessary for this result (the Δ*X.11* isoform of *Brca1* used in the study described above retains the RING domain). If Bard1 does not depend on its interaction with Brca1 to affect HRR efficiency, then transfection of the dominant-negative Bard1 protein into *gol/gol* cells should result in a decrease in the efficiency of HRR compared to that of *gol/gol* cells alone.

Normally, Brca1 appears to be escorted to (and retained in) the nucleus by Bard1; Brca1 appears to be similarly involved in Bard1 import and retention (Rodriguez, 2004). However, in *gol/gol* cells, lack of both the RING domain and (likely) the NES of Brca1 appears to result in normal nuclear import of the protein, as Brca1^{gol} is observed by immunofluorescence in both the nucleus and cytoplasm. This does not mean that the Brca1^{gol} protein is able to shuttle back and forth between the nucleus and cytoplasm, though, as the ΔX.11 form of the Brca1^{gol} protein would not be expected to be able to enter the nucleus (lacking, as it does, both the RING domain and the NLSs from exon 11) and should be observed in the cytoplasm. It might be worthwhile to investigate whether the Brca1^{gol} protein does shuttle in and out of the nucleus, and whether or not the ΔX.11 form of Brca1^{gol} is able to enter the nucleus.

7.6 THE E3 UBIQUITIN LIGASE ACTIVITY OF BRCA1-BARD1: A POSSIBLE FEEDBACK MECHANISM

Many RING-containing proteins are E3 ubiquitin ligases, involved in the 26S proteasome-mediated protein degradation pathway. Both BRCA1 and BARD1 have been shown to have E3 ligase activity, although it is increased when the two heterodimerize (Hashizume, 2001; Kentsis, 2002), and increased further by the formation of autopolyubiquitin chains on the heterodimer (Chen, 2002; Mallery, 2002). Much speculation has surrounded the E3 ubiquitin ligase capabilities of BRCA1 and BRCA1-BARD1 and whether substrates other than itself exist. The mouse protein Mouse double-minute 2 (Mdm2), a regulator of p53, is an E3 ligase which not only ubiquitinates p53, but also undergoes autoubiquitination to regulate its own stability (Fang, 2000). This suggests that BRCA1-BARD1 might have protein targets in addition to itself.

The *gol/gol* cells or mice should be excellent tools for investigating the targets of the Brca1-Bard1 E3 ubiquitin ligase, as the mutation in the RING domain means that neither Brca1 nor Brca1-Bard1 should be a functional E3 ligase. The human cancer cell line HCC1937 has been used to investigate BRCA1-related ubiquitin ligase activity in the past, but not only do these cells carry mutations besides the one in BRCA1, the RING domain is still intact in the mutated version of BRCA1 found in these cells, meaning that BRCA1-related E3 ligase activity may be partially functional in these cells. It would be especially interesting to investigate the involvement, if any, of Brca1 in the ubiquitination of RNA Pol II, an oft-suggested potential target of the Brca1-Bard1 E3 enzyme. Such studies may also reveal if Bard1 or Brca1 alone act as a ubiquitin ligase for other substrates.

Several groups have suggested that autoubiquitination may be a method of stabilizing the components of the BRCA1-BARD1 heterodimer; both mouse and *Xenopus* experiments have demonstrated that loss of one protein appears to result in a downregulation of the other (Joukov, 2001b; McCarthy, 2003). Data generated in this study suggests that the $Brca1^{gol}$ mutation is

upregulated or stabilized compared to wildtype Brca1, perhaps as a result of its inability to interact with Bard1. This result does not necessarily contradict the findings of previous studies, as the *gol* allele produces a mutant protein, while the other studies involved complete loss (or functionally relevant downregulation) of protein.

That autoubiquitination provides stability for the heterodimer is supported by *in vitro* data indicating that ubiquitin monomers are attached to BRCA1/BARD1 via a novel, Lys-6 linkage (different from the more common Lys-48 linkage observed on polyubiquitin chains of proteins destined for 26S proteasome-mediated degradation) (Wu-Baer, 2003). When ubiquitinated BRCA1/BARD1 is presented to the 26S proteasome, it is de-ubiquitinated, but not degraded (Nishikawa, 2004). Other groups have shown both that BRCA1 may not be degraded by the 26S proteasome, but by acid calpains or cathepsins instead, and that degradation of BRCA1 may occur in the nucleus (Blagosklonny, 1999; Choi, 2001). This could suggest that the Lys-6 ubiquitin chain targets the heterodimer to a different protease, or it may be a signal to target the heterodimer to the nucleus or cytoplasm, and not involved in stability or degradation.

The increased amount or increased stability of Brca1^{gol} may stem directly from the perturbation of a Brca1-Bard1 feedback loop. Before embarking on experiments to determine if Brca1 and Bard1 participate in a feedback loop, it would be worthwhile to first determine if Brca1 is more abundant or more stable in *gol/gol* cells. A time-course experiment following cyclohexamide treatment to block protein synthesis should help determine if the half-life of Brca1^{gol} protein is extended compared to wildtype Brca1. Further experiments might include blocking acidic protease and/or 26S proteasome activity, monitoring ubiquitin chain formation on Brca1^{gol}, and investigating the stability, ubiquitination status, and amount of Bard1 protein in *gol/gol* cells.

7.7 POTENTIAL FUTURE EXPERIMENTS WITH THE *gol* ALLELE

One of the most difficult problems when faced with the enormous volume of scientific literature published about BRCA1 biology is trying to link together the many interactions that the BRCA1 protein appears to be involved in. One advantage of having a viable mutant allele lacking a specific domain is that it affords a chance to investigate what that domain of the protein does or does not interact with, without using transgene overexpression or yeast 2-hybrid assays. Such investigations should include additional immunolocalization experiments, involving proteins such as Bard1 and Rad51, and perhaps proteins such as PCNA (following DNA damage). A microarray assessment of *gol/gol* cellular mRNA before and after DNA damage to look at gene induction following damage might also yield interesting results.

Experiments which extend the functional studies described in previous chapters are easily envisioned. It would be worthwhile to determine if a transcriptional repressor element is present in intron 2 of the mouse *Brca1* gene. This could be done via a novel BAC system constructed by a colleague, Haydn Prosser. His system is designed to allow BACs to be introduced into ES cells in a defined location. BACs can now be modified fairly easily through recombineering, and by recombineering a series of BACs carrying mouse *Brca1* with a series of intron 2 deletions, the location of this putative repressor could be located. The efficacy of the repressor could then be confirmed using a reporter gene assay. It would also be worthwhile to determine where the initiation site of the *gol* transcript is – and if more than one is utilized.

The DNA damage phenotypes of *gol/gol* cells could be studied much more extensively; it would be quite interesting to look by SKY or karyotyping analyses to assess the amount of genomic rearrangement in *gol/gol* MEFs or ES cells after a number of passages in culture, or following DNA damage. It should also be possible to determine if the lack of difference in colony-forming ability between *gol/gol* and wildtype ES cells following UV exposure is due to efficient repair or a greater tolerance of damage. Additionally, by

synchronizing either MEFs or ES cells, it could be determined if *gol/gol* cells are susceptible to DNA damage at certain phases of the cell cycle. The results of such an experiment may be helpful in determining whether the slight decrease in HRR efficiency in these cells is functionally relevant, as HRR and NHEJ are generally used at different points in the cell cycle (Takata, 1998; Wang, 2001b).

A mouse *gol* model is in development, and, if viable, is potentially quite interesting with many future applications. For a start, the viability (in terms of expected Mendelian frequency) and/or the fertility of the mice will be investigated. Should the homozygotes be viable, then of course tumorigenic studies will be performed on them. In conjunction with this, might be interesting to see how *gol/gol* mice respond to MMC treatment or γ-irradiation as tumourigenic accelerants. If it is revealed at a later date that expression of mutant *Brca1* in a specific tissue or cell-type would be advantageous, a conditional allele of *gol* exists (*c2*) which could be used. The analysis of any resulting tumours by microarray for changes in expression and possibly for the loss or gain of protein products may also yield useful information.

Generating a *gol/gol, Bard1*$^{-/-}$ model would also be interesting, and at the very least may reveal whether the *Bard1* deficiency is lethal because of the role of Bard1 as nuclear chaperone/anchor to Brca1. If the *gol/gol* mice are not viable, then it might be worth trying to cross the allele onto the *Bard1* knockout background, to see if the two mutations might rescue one another.

7.8 SOME FINAL WORDS

In the mouse, Brca1 appears to contribute to carcinogenesis through its role as a caretaker tumour-suppressor. Although there are several mouse models which provide a variety of models for *BRCA1*-related breast cancer, to date it has been equally worthwhile to investigate the DNA damage response and cell cycle-related functions of Brca1 in cells. The involvement of Brca1 in such processes appears to be well-conserved across species, and in this regard, the mouse makes an excellent model for the human. The original goal of this project was to generate a tool for investigating the function of *Brca1* in cells, and the *gol/gol* cells can indeed be regarded as such a tool. It

is hoped that the *gol* allele will add to the many studies of Brca1 function, leading to a greater understanding of its roles in the cell and how the loss of these actions eventually leads to tumourigenesis.

After nearly ten years of study, involving hundreds of laboratories, it may seem that little progress has been made toward these goals, but the study of *BRCA1*-related breast cancer is not terribly different from that of most cancers. It may be argued that the majority of cancer therapies in use today – indeed, the majority of therapies for any human disorder – do not directly exploit knowledge about the molecular mechanisms involved. This appears disheartening, but taken from another angle, it suggests that huge strides forward will be made in the near future, as the molecular findings from the past and present are translated from understanding into therapies and advanced prevention and diagnostic techniques. Glimpses into this rosier future are given by cancer drugs such as Gleevec, an inhibitor of the kinase product of the BCL-ABL fusion (resulting from translocation) found in many leukaemia patients, or Herceptin, which inhibits growth of breast cancer cells overexpressing the *HER2/neu/ErbB2* gene (reviewed in Shawver, 2002). These therapies were developed using knowledge of the role of specific proteins in specific cancers, and such “smart drugs” may completely transform cancer therapeutics. Added to the rapidly-increasing outpouring of information about molecular interactions and functions of virtually every gene, transcript, and protein in the genome, this makes the next ten years a very exciting prospect.

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