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): <u>*Puro*-bpA (no promoter)</u> was generated using a *Sal*I/*Xho*I$_{partial}$ –digested pPGK-*Puro*-bpA vector; a 4.0 kb fragment was isolated and re-ligated. <u>Reversed-PGK-*Puro*-bpA (revPGK-*Puro*-bpA)</u> 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*$^{b\text{-}m2}$) 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 electroporation)	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*$^{\mathrm{Brdm2}}$ 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 $2\mathrm{x}10^5$ *–/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.).