Chapter 2

Materials and Methods

2.1 Embryonic stem cell lines

2.1.1 Wild-type cell lines

AB2.2 is derived from a 129S6 blastocyst (McMahon and Bradley, 1990). The cell line carries an inactivating mutation in the *Hprt* gene on the X chromosome.
AB1 is derived from the same mouse strain as AB2.2, but has an active *Hprt* gene.
JM8 is derived from a C57BL/6N blastocyst. Feeder-independent (JM8.N4) and dependent (JM8.F6) subclones are available. I derived JM8A3 from JM8.F6 by fixing the naturally occurring *nonagouti* (*a*) coat colour mutation (Pettitt *et al.*, 2009); this cell line is used as wild type in some experiments.

2.1.2 *Blm*-deficient cell lines

NN5 was derived from AB2.2 cells by gene targeting (Luo *et al.*, 2000; Guo *et al.*, 2004). These cells are compound heterozygotes at the *Blm* locus, genotype $Blm^{tm3Brd/tm4Brd}$. The m4 allele is a deletion of exon two. The m3 allele results from an insertion event followed by Cre recombinase treatment, the net result being a duplication of exon 3.
NRB2 and RECE8 are NN5 cells containing a Cre-ERT2 gene integrated by gene targeting at the *Rosa26* locus (Figure 2.1). I used the same targeting method described in Vooijs *et al.* (2001), although I used a *bsd*-expressing version of the targeting vector obtained from David Adams. The two lines were derived from the same targeting.

Figure 2.1: Targeting NN5 cells with a *Rosa26*:Cre-ERT2 construct. Digest, probe as in Vooijs *et al.* (2001). Left, NRB2; right NN5.

$\boldsymbol{Blm}^{e/e}$ was derived from JM8.F6 by Amy Meng Li (Li, 2010). The *Blm* locus is homozygously targeted in these cells, and incorporates a blasticidin S deaminase (*bsd*) selectable marker gene and an enhanced green fluorescent protein gene (*EGFP*), both of which are constitutively expressed. These cells display the increase in sister chromatid exchanges (SCEs) characteristic of Bloom syndrome and do not express detectable Blm protein.

2.1.3 Other mutant cell lines

$\boldsymbol{Xrcc4}^{-/-}$ **and** $\boldsymbol{Xlf}^{\Delta/\Delta}$ are derived from the TC1 wild type cell line (129S7 strain) (Zha *et al.*, 2007). These cells were a kind gift from Fred Alt and Shan Zha (Children's Hospital, Harvard Medical School).

2.2 Cell culture

2.2.1 Culture conditions

ES cells were maintained in DMEM supplemented with 15% serum, 2 mM L-glutamine and 100 μM β-mercaptoethanol (M15 medium) on a layer of irradiated SNL76/7 feeder fibroblasts as previously described (Ramírez-Solis *et al.*, 1993). Medium was changed daily. For JM8 and its derivatives, recombinant mouse leukaemia inhibitory factor (LIF) was added to growth medium at 100 U/ml. For routine passaging cells were treated with 0.1% trypsin-EDTA in phosphate buffered saline (PBS) for 15 minutes (10 minutes for JM8 derivatives), quenched with an equal volume of M15 medium, clumps disrupted by pipetting and cells then transferred to a fresh plate pre-fed with M15.

2.2.2 Selective media

Drugs used for selection and their concentrations are listed in Table 2.1. For convenience, several abbreviations for drug-containing M15 media are used as follows: **DBL**, G418 (200 μg/ml) and Puromycin (3 μg/ml). **HGFL**; HAT, G418 and FIAU [L—LIF]. **HTGL**; HT (hypoxanthine and thymidine, i.e. HAT without aminopterin) and G418.

Drug	Concentration	Purpose
G418 (Geneticin)	180–200 μg/ml	Selects for *neo* expression
Puromycin	3 μg/ml	Selects for *puro* expression
Blasticidin S	10 μg/ml	Selects for *bsd* expression
HAT	0.1 mM/0.4 μM/16 μM	Selects for *Hprt* expression
6-Thioguanine	10 μM	Selects against *Hprt* expression
FIAU	200 nM	Selects against hsvTK (ΔTK)
Bleomycin	0.1–1 μg/ml	Causes double strand breaks

Table 2.1: Drugs used in selective media and concentrations. HAT—Hypoxanthine/Aminopterin/Thymidine mixture; FIAU—1-(2-deoxy-2-fluoro-1-D-arabinofuranosyl)-5-iodouracil

Mechanism of resistance

Most of the resistance genes encode an enzyme with activity that metabolises the associated drug, rendering it non-toxic. The exception is selection involving *Hprt*. HAT medium, a mixture of hypoxanthine, aminopterin and thymidine is used to select for *Hprt* (positive selection). There are two cellular pathways for guanine and adenine (the purine bases in DNA) synthesis, the *de novo* pathway which synthesises purines from simple metabolites, and the salvage pathway, which recovers purine ring compounds from other pathways. *Hprt* encodes an enzyme in the salvage pathway, hypoxanthine/guanine phosphoribosyltransferase, which adds a ribose sugar and phosphate to recovered bases to form a nucleotide that can be incorporated into RNA or reduced to form a deoxyribonucleotide for DNA synthesis. The salvage pathway can support cell growth and division on its own if the *de novo* pathway is blocked, provided there are enough purines around for salvage, but in this situation *Hprt* becomes an essential gene. This is the basis of HAT selection: aminopterin is a small molecule inhibitor of dihydrofolate reductase (DHFR), a key enzyme in the *de novo* pathway. Therefore, when cells are grown in aminopterin they are dependent on the salvage pathway, and thus on a functional copy of *Hprt*. A high concentration of hypoxanthine is included as a substrate for Hprt. After HAT selection, the medium is supplemented with hypoxanthine for two further days (I use 1× HT supplement, Invitrogen), to allow DHFR activity to recover. Although HAT and HT media contains thymidine, it is not relevant in this case; it is included to enable a similar selective strategy to be used with the pyrimidine synthesis pathway and the thymidine kinase gene.

Selection against *Hprt* function uses 6-thioguanine (6-TG). This is metabolised to 6-thioguanosine by Hprt, which can be incorporated into DNA. This is recognised by the mismatch repair machinery, leading to a persistent DNA damage response that eventually results in cell death. FIAU works on a similar basis as a toxic uracil mimic (dUMP can be metabolised to dTMP and incorporated into DNA).

2.2.3 Transfection of ES cells

Electroporation was carried out as described previously (Ramírez-Solis *et al.*, 1993). Typically, a suspension of 1×10^7 cells in 0.9 ml PBS, pre-mixed with DNA, was electroporated at 230 V, 500 μF in a BioRad GenePulser. After incubation at room temperature for five minutes, cells were transferred to a plate with feeder cells and M15 medium.

Lipofection using Lipofectamine 2000 (Invitrogen) was either done using 90% confluent adherent ES cells using the manufacturer's protocol or, with generally better results, using trypsinised cells in suspension. Cells were fed two hours prior to transfection. One hour later, Lipofectamine-DNA complexes were prepared as recommended by the manufacturer and left to incubate at room temperature (100 μl total volume for a 24-well plate). Cells were trypsinised and resuspended in OptiMEM (Invitrogen, 500 μl for a 24-well plate, around 500,000 cells), added to a fresh plate and mixed with the Lipofectamine-DNA solution. After incubation at 37° for three hours, 1 ml M15 medium was added. Cells were usually passaged the next day to a larger plate. Amaxa transfections or lipofections using the Transmessenger reagent (Qiagen) were per manufacturers' protocols.

2.2.4 Cellular analysis

Flow cytometry

For flow cytometry of live cells, cells were harvested by trypsinisation and resuspended in PBS with 1% FCS. The suspension was filtered through a 30 μm mesh (Partec CellTrics) immediately prior to flow

cytometry. A Beckman-Coulter FC-500 was used for flow cytometry and data analysed using Flo-Jo software.

For DNA content analysis, cells were fixed by pipetting a small volume of cell suspension in PBS directly into 5 ml 70% ethanol at –20°C. After fixing overnight at –20°C, the resulting nuclei were resuspended in PBS containing 2 μg/ml propidium iodide and 0.5 mg/ml RNAse A, and incubated at room temperature to digest RNA.

Growth analysis

To stain colonies, I rinsed plates once in PBS and added a small amount of 1% (w/v) methylene blue in 70% ethanol. After 15 minutes I rinsed plates by submerging several times in tap water, and left to destain in water overnight. For measurements of cell viability I used the MTT test. MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was dissolved in PBS, with sonication, to make a 5 mg/ml stock. Cells were fed with M15 medium and one tenth of the volume of MTT solution added two hours later. After two hours, a purple precipitate forms in actively respiring cells. The precipitate was dissolved in 1:1 DMSO:Ethanol by shaking the plate for 2 hours at room temperature. Absorbance at 540 nm was measured in a plate reader, and a background reading at 620 nm subtracted.

Preparation of metaphase spreads

Actively growing cells in a 6-well plate (fed two hours previously) were treated with demecolcine (1 μg/ml) for at least one hour. Cells were harvested by trypsinisation and washed with PBS. The suspension was centrifuged and cells resuspended in the residual PBS in 14 ml round-bottom Falcon tubes. Five millilitres of 0.56% KCl was added and cells incubated at room temperature for seven minutes to swell cells. The cells were spun at 400×g for five minutes, the supernatant decanted and resuspended in the residual KCl solution by tapping the tube. To fix, 5 ml of 40% methanol:10% acetic acid fixative was added dropwise, with constant agitation using a vortex mixer set to a low setting. The preparation was centrifuged as above and this fixing process repeated one. After a final spin, the nuclei were resuspended in 200 μl of fixative and dropped onto slides to make chromosome spreads. Fixed nuclei were stored in fixative at -20°C.

2.2.5 Isolation of nucleic acids and proteins

Preparation of DNA for enzyme digestion

From 96-well plates, I followed the protocol described in Ramírez-Solis *et al.* (1993). For larger cultures, I harvested cells by trypsinisation, washed with PBS and lysed overnight in ES cell lysis buffer at 55°C. The next day, an equal volume of isopropanol was added to precipitate DNA. The aggregate was retrieved using a sealed glass capillary, rinsed in 70% and 100% ethanol and dried for five minutes at room temperature. DNA was redissolved in 5 mM Tris-HCl pH 8.0 or 10 mM Tris-HCl, 0.1 M EDTA pH 8.0 and stored at 4°C.

Preparation of cell lysates for PCR

For preparation of lysates directly from colonies, I picked colonies into 50 μl trypsin as usual, quenched the trypsin with an equal volume of M15 medium and pipetted to form a single cell suspension. Eighty microlitres of this was transferred to a 96-well plate for expansion. To the remainder, I added 180 μl PBS and span the plate at 800 ×g for five minutes. The supernatant was removed and cells resuspended in a tiny drop of PBS. Fifty microlitres of PCR lysis buffer (1× PCR buffer with 0.45% NP-40, 0.45% Tween-20 and proteinase K, McMahon and Bradley (1990)) were added. The plate was incubated in a humid atmosphere overnight at 55°C, and heated to 95°C for 20 minutes the next day to denature the proteinase K. Up to $\frac{1}{5}$ of the PCR volume was used as template.

Preparation of RNA

RNA was prepared using Trizol (Invitrogen) using the manufacturer's protocol.

Preparation of lysates for Western blotting

After washing in PBS, cells were lysed in ELB buffer (150 mM NaCl, 50 mM HEPES pH 7.5, 5 mM EDTA, 0.1% NP-40 including Complete protease inhibitor cocktail (Roche); 10^6 cells per ml) on ice for 30 minutes. Tubes were spun briefly to pellet debris, and the supernatant removed and stored at –20°C. Protein was quantified using D_C reagent (BioRad) with BSA as a standard.

2.3 ES cell genotyping

2.3.1 PCR and long range PCR

Conventional PCR used ThermoStart polymerase (Thermo Scientific). For long range PCR to confirm gene targeting, I used Extensor PCR (Thermo Scientific) with a protocol as follows: 92°C 2 minutes; 10 cycles of: 92°C 30 s, 55°C 30 s, 68°C 4 minutes; 20 cycles of: 92°C 30 s, 55°C 30 s, 68°C 4 minutes plus 10 seconds per cycle; 5 minutes 68°C. For genotyping the *Rosa26*:ERT2-iCre-ERT2 targeting I used LA Taq (Takara) as per manufacturer's instructions.

2.3.2 Mapping transposon integration sites by splinkerette PCR

Splinkerette PCR is a linker based PCR method to amplify a product where the sequence is only known at one end, i.e. the transposon (Devon *et al.*, 1995). The splinkerette is a double-stranded adaptor oligonucleotide that contains an unpaired region, in which one strand forms a hairpin with itself. Genomic DNA is digested with a restriction enzyme, usually a frequent cutter with a four base pair recognition site, and the splinkerette adaptors ligated. The ligation products are then used as template for PCR using one primer extending outwards from the transposon sequence, and one of identical sequence to the unpaired region of the non-hairpin strand of the splinkerette. This second primer is of no use until its complement has been synthesised by extension of the transposon primer (Figure 2.2, (Li *et al.*, 2010)). This ensures that only fragments that contain the transposon sequence are amplified. A further nested PCR step also improves specificity.

To prepare Splinkerette adaptors, I combined 150 pmol of each oligonucleotide in 100 μl of water and heated to 95°C for five minutes. The solution was allowed to cool slowly to room temperature, then stored at –20°C. I carried out restriction digests in 96-well plates overnight as for Southern blots, or in tubes using 5 μg of genomic DNA, using a total volume of 50 μl. I usually used *Sau*3AI or *Bfu*CI restriction enzymes, both of which leave a 5′ GATC overhang. After digestion, the enzyme was heat inactivated and 1.5 μl used in a ligation reaction with 2.5 μl adaptor solution in a total volume of 10 μl. Ligation was at 16°C overnight, and the reaction was heat inactivated the next day. One microlitre was used as template for PCR were carried out using ThermoStart polymerase, in a volume of 25 μl with 2 mM $MgCl_2$. Cycling conditions were as follows: 94°C, 30 s; 62°C 30 s; 72°C 90 s, 30 cycles followed by five minutes final extension at 72°C. One microlitre was used as template for the secondary PCR, using the same conditions. Splinkerette and primer sequences are given in Appendix B.

2.3.3 Southern blot

Probes were designed to be at least 300 bp, preferably 800-1000 bp long. For probes hybridising to genomic sequence, RepeatMasker[1] was used to exclude repetitive regions from the probe. PCR products were amplified from BACs where available, or by two rounds of PCR from genomic DNA. For internal transposon probles, restriction fragments of plasmids were used. All probes were gel purified. **Labelling** used the random primer method using α^{32}P-dCTP (PrimeIt II kit, Agilent). Twenty five nanograms of probe were used in a 50 μl labelling reaction; one labelling reaction was used for up to three hybridisations carried out in parallel. Five hundred picograms of 1 kb λ ladder DNA (Invitrogen) was included in the labelling reaction to show the molecular weight markers. **DNA** was prepared as above. Either an entire 96-well plate (following the procedure described in Ramírez-Solis *et al.* (1993)), or 5–10 μg, was digested overnight with 30 units of restriction enzyme in the appropriate buffer (50 μl volume). **Electrophoresis and transfer** used 0.6–0.8% agarose gels run for at least five hours or overnight at low voltage in 1X TAE buffer. Five nanograms of 1 kb λ ladder was run as a marker; a larger amount was typically run in a lane as far as possible from the samples for visualisation with ethidium bromide staining. Gels were soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 1–2 hours. After saturation of the gel with denaturing solution, it was placed upside down on a sheet of cling film and the following placed on top: one sheet Hybond XL charged nylon membrane (GE healthcare), two sheets filter paper (Whatman), paper towels to a height of 10–15 cm. The entire transfer apparatus was covered with cling film, and the gel tray placed on top as a weight. This was left overnight to transfer. The following day, the membrane was washed in 2× SSC for five minutes and baked at 80°C for at least 30 minutes to dry out. **Hybridisation.** The buffer used for hybridisations was: 1.5× SSPE, 1% SDS, 1% (w/v) skimmed milk powder (final concentrations). Sheared, freshly boiled, salmon sperm DNA was added just before prehybridisation to a final concentration of 200 μg/ml.

[1] http://www.repeatmasker.org

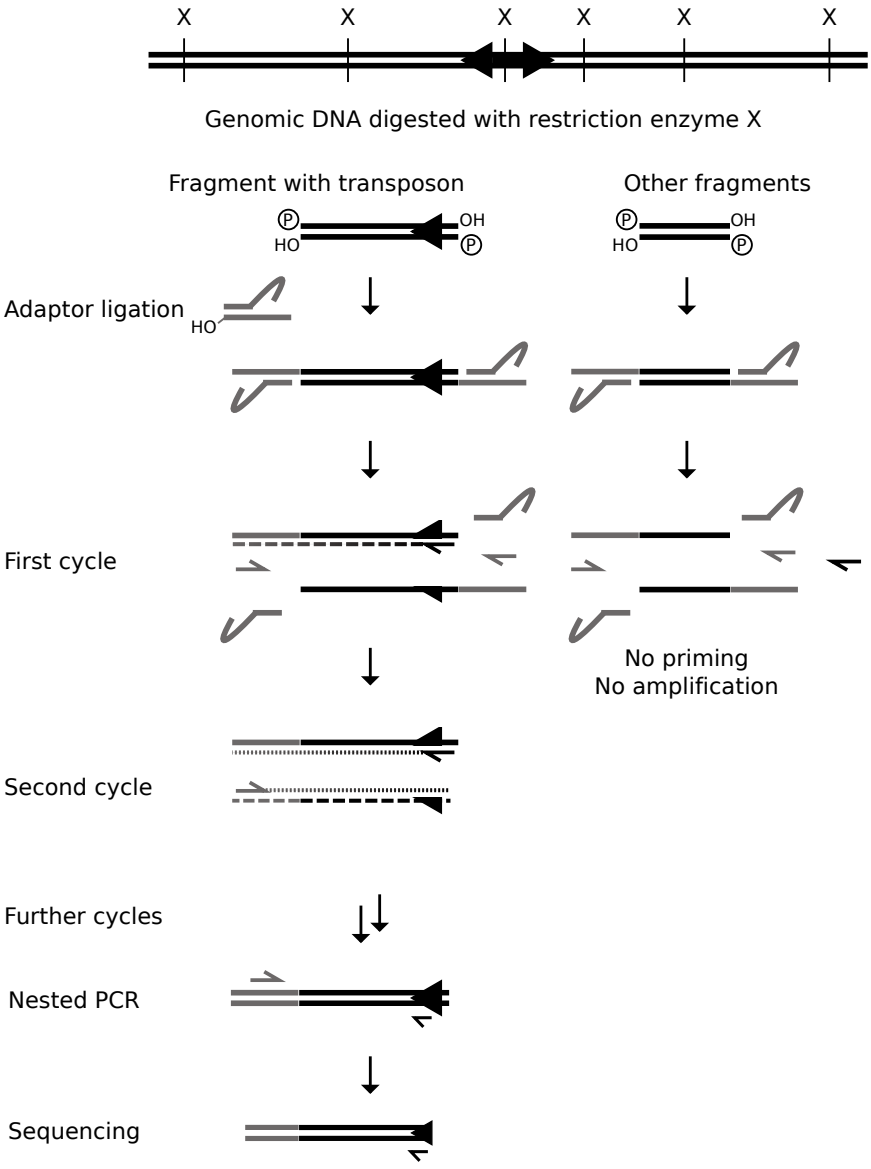

Figure 2.2: Splinkerette PCR method

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Eight millilitres was typically used for a membrane of 30 × 10 cm. Prehybridisation was carried out for one hour at 68°C in a rotisserie oven, after which the (boiled) probe was added directly to the prehybridisation buffer. Hybridisation was at 68°C overnight.
Washing. The membrane was briefly rinsed twice at room temperature in 2× SSC, 1% SDS. This was followed by two washes at 65°C for 30 minutes each. Membranes were rinsed briefly in 2× SSC, sealed in bags and exposed to film for 1–5 days at –80°C.

2.3.4 RT-PCR

Three micrograms of RNA was used for reverse transcription (SuperScript II, Invitrogen) using oligo-dT primers, following the manufacturer's protocol. The reaction was diluted 1:5, and 1 μl used as template for PCR using standard conditions.

2.3.5 Western blot

Proteins were separated on pre-cast 4–12% Bis-Tris PAGE gels (NuPAGE, Invitrogen) using MOPS buffer. The proteins were transferred to a PVDF membrane. The membrane was blocked in PBST buffer (0.1% Tween-20) with 5% (w/v) skimmed milk for one hour at room temperature. The primary antiserum, diluted 1/200 in blocking buffer, was applied in a total volume of 2 ml, held on the protein surface of the blot by surface tension, and incubated overnight at 4°C. The membrane was washed three times in PBST prior to incubation with horseradish peroxidase (HRP) conjugated secondary antibody (1/1000 dilution) for one hour at room temperature. ECL+ chemiluminescence reagents were used for visualisation.

2.4 Molecular biology

2.4.1 Recombineering

Principle

Recombineering refers to manipulation of DNA in bacteria using recombination. Most commonly used lab strains of *E. coli* have a *recA* mutation. The *recA* gene product is homologous to eukaryotic Rad51, and forms a single stranded protein-DNA filament that begins the process of homologous recombination. This pathway needs to be knocked out to allow high copy number plasmids to be stably maintained without recombining with each other. Therefore, *recA* mutant bacteria form a stable environment to maintain and propagate plasmids. DNA manipulations (restriction digests, ligation etc.) are usually carried out *in vitro* and the products used to transform bacteria.

The recombineering method takes a different approach, and is essentially analogous to gene targeting in bacteria. The method works by transiently rescuing the *recA* mutation. At this point, homologous sequences that are present in the bacterium recombine with high frequency. Thus by designing suitable targeting constructs, BACs and plasmids can be manipulated. Importantly, as few as 30 nt of homology is sufficient for recombination, so these constructs can be easily synthesised as oligonucleotides, or tailed PCR primers.

I used the **EL350**, or its derivative **SW106**, strain for recombineering (Lee *et al.*, 2001; Warming *et al.*, 2005). EL350 contains an integration of a defective λ prophage, encoding the phage genes *exo*, *bet* and *gam*. The *exo* and *bet* genes encode a 5′ to 3′ exonuclease that resects DNA ends, exposing single stranded DNA to which *bet*, which substitutes for *recA*, can bind. The *gam* gene product is an inhibitor of the bacterial RecBCD nuclease complex, and when expressed prevents degradation of linear DNA (i.e. the introduced targeting construct) by RecBCD. The phage recombination genes are under the control of a mutant cI promoter that is repressed at 32°C and de-repressed at 42°C. Therefore, bacteria for recombineering are always grown at 32°C (there is some leaky expression of the recombination operon at 37°C), and heat shocked at 42°C immediately prior to transfection of the targeting construct.

Protocol

I typically grew 25 ml bacterial cultures, inoculated from an overnight starter culture, in baffled conical flasks at 32°C until an OD_{585} of 0.4–0.6 was reached. Then the culture was split in two, and one half grown in a 42°C shaking waterbath for 15 minutes, with the other (control) half remaining at 32°C. Both flasks were then transferred to an ice bath and swirled for five minutes to cool. To make electrocompetent cells, I then washed twice with ice cold distilled water (or 10% glycerol) in 14 ml round bottom Falcon tubes. Using round bottom tubes allows the bacteria to be resuspended very gently by swirling the tube in an ice-water slush. Electrocompetent cells were electroporated at 1.8 kV in a BioRad GenePulser, using 50 μl cell suspension in a 0.1 cm cuvette. Typically 1–10 ng of plasmid or targeting construct was used for transformation, and 50–100 ng for BAC. After electroporation 900 μl SOC medium was added, the culture transferred to a 14 ml Falcon tube and recovered in a 32°C shaking

incubator for at least one hour prior to plating.

2.4.2 Conventional cloning

Plasmid manipulation was carried out using standard procedures, using restriction endonucleases, antarctic or calf intestinal phosphatases, T4 polynucleotide kinase and T4 ligase purchased from NEB (Maniatis *et al.*, 1982). For gel purifications I used a kit from ZymoClean. Plasmids were usually maintained in DH5α *E. coli* purchased as chemically competent cells from Invitrogen and following their protocol for transformation. Ampicillin selection (*bla* gene) used 100 μg/ml ampicillin in LB or 2×TY medium, blasticidin selection (for *EM7-bsd*) using 50 μg/ml in low salt LB (Invivogen).