2 Material and methods

2.1 Vectors

2.1.1 Vectors for induced mitotic recombination

pL330 (*Hprt* MΔ3') and pL341 (*Hprt* MΔ3') are kind gifts from Dr. Pentao Liu (Liu, Jenkins et al. 2002). These vectors contain *Neo*^{R} and *Puro*^{R} selection markers flanked by three lox site variants: *lox*5171, *lox*2272 and either *lox*66 (*Hprt* MΔ3') or *lox*71 (*Hprt* MΔ3') sites. A wild-type *lox*P site is generated by site-specific recombination between *lox*66 and *lox*71 sites. Because the wild-type *lox*P site will be used for regional trapping, *lox*66 and *lox*71 sites need to be deleted from these mitotic recombination vectors. Several vectors were constructed for this purpose.

pWW15 (*Pol II-Neo-bpA* cassette):
pL341 was cut with *Hin*dIII and *Not*I, a 2.2 kb fragment was gel purified and digested again with *Spe*I, a 1.8 kb fragment was gel purified and cloned into pBluescript (pBS) plasmid (Stratagene) digested with *Hin*dIII and *Spe*I to make pWW15.

pWW22 (*PGK-Puro-bpA* cassette):
1) pL330 was digested with *Eco*RI and *Hin*dIII sequentially, a 0.5 kb fragment was gel purified; 2) pL330 was digested with *Eco*RI and *Hin*dIII sequentially, a 1.2 kb fragment was gel purified and digested again with *Spe*I, 1.0 kb fragment was gel purified; 3) pBS was digested with *Eco*RI and *Spe*I, a 2.9 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way-ligation to make pWW22.

pWW23 (*PGK*-5' *Hprt* cassette):
1) pL341 was digested with *Hin*dIII and *Bgl*II sequentially, a 0.7 kb fragment was gel purified; 2) pL341 was digested with *Hin*dIII and *Bgl*II sequentially, a 1.0 kb fragment was gel purified and digested again with *Spe*I, a 0.8 kb fragment was gel purified; 3) pBS was digested with *Hin*dIII and *Spe*I, a 2.9 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way-ligation to make pWW23.

pWW24:
A variant polylinker site *Hin*dIII-*Spe*I-*Xba*I-*Nde*I-*Pst*I-*Eco*RI-*Not*I (pWW24) was generated by cutting pBS with *Hin*dIII and *Not*I and ligating to a pair of complementary oligonucleotids, Oligo-(*Hin*dIII-*Not*I)-For and Oligo-(*Hin*dIII-*Not*I)-Rev (Table 2-1).

pWW37 (multi *lox* sites):
A polylinker site with *lox5171*, *lox2272* and a *FRT* site (pWW37) was generated by cutting pWW24 with *Hin*dIII and *Xba*I and ligating to a pair of complementary oligonucleotids, *lox*P-(*Hin*dIII-*Xba*I)-For and *lox*P-(*Hin*dIII-*Xba*I)-Rev (Table 2-1).

pWW43:
1) pL341 was linearized by *Not*I first and then partially digested with *Hin*dIII, a 4.5 kb fragment was gel purified; 2) pWW15 was digested with *Hin*dIII and *Spe*I, a 2 kb fragment was gel purified; 3) pWW37 was digested with *Spe*I and *Not*I, a 0.2 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way ligation to make pWW43.

pWW48 (multi *lox* sites-3' *Hprt* cassette):
1) pWW37 was digested with *Sal*I and *Eco*RI, a 0.2 kb fragment was gel purified; 2) pWW22 was digested with *Eco*RI and *Hin*dIII, a 0.5 kb fragment was gel purified; 3) pWW22 was digested with *Hin*dIII and *Spe*I, a 1 kb fragment was gel purified; 4) pWW37 was digested with *Spe*I and *Nde*I, a 0.2 kb fragment was gel purified; 5) pL330 was digested with *Sal*I and *Nde*I, a 4.9 kb fragment was gel purified. The five fragments mentioned above were ligated together in a five-way ligation to make pWW48.

Table 2.1: Primers used for oligo ligation.

Primer Pair Name	Description	Forward Primer Name	Forward Primer Sequence	Reverse Primer	Reverse Primer Sequence	Plasmid
Oligo-(*Hin* dIII-*Not* I)	A variant polylinker site *Hin* dIII-*Spe* I-*Xba* I-*Nde* I-*Pst* I-*Eco* RI-*Not* I	Oligo-(*Hin* dIII-*Not* I)-For	5'-AGC TTA TCC ACT AGT CAC GGC CGC CAA AAG CGC TCT GAA GTT CCT ATA CTT TCT AGA GAA TAG GAAC TTC GGA ATA GGA ACT TCA AAG CGC ATA TGT CTG CAG GAA TTC GAT GAT CCA CTA GAG C-3'	Oligo-(*Hin* dIII-*Not* I)-Rev	5'-GGC CGC TCT AGT GGA TCA TCG AAT TCC TGC AGA CAT ATG CGC TTT GAA GTT CCT ATT CCG AAG TTC CTA TTC TCT AGA AAG TAT AGG AAC TTC AGA GCG CTT TTG GCG GCC GTG ACT AGT GGA TA 3'	pVWV24
lox P-(*Hin* dIII-*Xba* I)	A polylinker site with *lox5171* , *lox2272* and *FRT* sites	*lox* P-(*Hin* dIII-*Xba* I)-For	5'-AGC TTA TCC ACT AGT TAG GGA TAA CAG GGT AAT TCT AGT ATA ACT TCG TAT AAT GTG TAC TAT ACG AAG TTA TTC TAG TAT AAC TTC GTA TAA AGT ATC CTA TAC GAA GTT ATT-3'	*lox* P-(*Hin* dIII-*Xba* I)-Rev	5'-CTA GAA TAA CTT CGT ATA GGA TAC TTT ATA CGA AGT TAT ACT AGA ATA ACT TCG TAT AGT ACA CAT TAT ACG AAG TTA TAC TAG AAT TAC CCT GTT ATC CCT AAC TAG TGG ATA-3'	pVWV37
lox P-(*Xba* I-*Sal* I)	A polylinker site with *lox* P site	*lox* P-(*Xba* I-*Sal* I)-For	5'-CTA GAT AAC TTC GTA TAG CAT ACA TTA TAC GAA GTT ATG-3'	*lox* P-(*Xba* I-*Sal* I)-Rev	5'-TCG ACA TAA CTT CGT ATA ATG TAT GCT ATA CGA AGT TAT-3'	pVWV144
lox P-(*Eco* RI-*Hin* dIII)	A polylinker site with *lox* P site	*lox* P-(*Eco* RI-*Hin* dIII)-For	5'-AAT TCA TAA CTT CGT ATA GCA TAC ATT ATA CGA AGT TAT-3'	*lox* P-(*Eco* RI-*Hin* dIII)-Rev	5'-AGC TAT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT ATG-3'	pVWV205

pWW49 (5' *Hprt*-multi *lox* sites cassette):
1) pWW43 was partially digested with *Nde*I and then digested with *Sal*I, a 5 kb fragment was gel purified; 2) pWW23 was partially digested with *Spe*I and then digested with *Sal*I, a 1.5 kb fragment was gel purified. 3) pWW37 was digested with *Spe*I and *Nde*I, a 0.2 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way-ligation to make pWW49.

pL325 is a gift from Dr. Pento Liu. To make this plasmid, a *D11Mit71* genomic fragment from pBZ84 (Zheng, Sage et al. 2000) was cloned into a vector containing the MC1-*tk* negative selection marker. A polylinker containing *Xho*I and *Not*I digestion sites was used to replace a 0.8 kb *Nco*I fragment. A selection cassette can be cloned into this polylinker to make a *D11Mit71* targeting vector.

pWW74 (multi *lox* sites-3' *Hprt* cassette, *D11mit71* targeting vector):
1) pL325 was digested with *Cla*I and *Not*I, a 6 kb fragment was gel purified; 2) pL325 was digested with *Cla*I and *Xho*I, a 4 kb fragment was gel purified; 3) pWW48 was digested with *Sal*I and *Not*I, a 3.9 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way-ligation to make pWW74.

pWW75 (5' *Hprt*-multi *lox* sites cassette, *D11mit71* targeting vector):
1) pL325 was digested with *Cla*I and *Not*I, a 6 kb fragment was gel purified; 2) pL325 was digested with *Cla*I and *Xho*I, a 4 kb fragment was gel purified; 3) pWW49 was digested with *Sal*I and *Not*I, a 3.8 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way-ligation to make pWW75.

2.1.2 Vectors for *E_2DH* end point targeting

pWW63 (*PGK* promotor):
pWW48 was digested with *Eco*RI and *Bgl*II, a 0.5 kb fragment was gel purified and cloned into pBS digested with *Eco*RI and *Bam*HI to make pWW63.

pWW144 (*PGK-lox*P):
1) pWW63 was digested with *Hin*dIII and *Xba*I, a 0.5kb fragment was gel purified; 2) a pair of complementary oligonucleotids, *lox*P-(*Xba*I-*Sal*I)-For and *lox*P-(*Xba*I-*Sal*I)-Rev, were annealed together (Table 2-1); 3) pBS was digested with *Hin*dIII and *Sal*I, a 2.9 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way-ligation to make pWW144.

pL313 is a kind gift from Dr. Pentao Liu. It contains a *PGK-EM7-Bsd-bpA* cassette. This cassette can be selected both in *Escherichia coli* (75 μg/ml) and in eukaryotic cells (10 μg/ml) using Blasticidin S HCl.

pWW146 (*PGK-lox*P-*EM7-Bsd-bpA*):
1) pWW144 was digested with *Hin*dIII and *Sal*I, a 600 bp fragment was gel purified; 2) pL313 was partially digested with *Xho*I, and then digested with *Hin*dIII, a 3.7 kb fragment was gel purified. The two fragments were ligated together to make pWW146.

pL10 and pL11 are two vectors that contain 5' and 3' genomic insert of E_2DH locus, respectively (Liu, Zhang et al. 1998).

pWW183 (E_2DH targeting vector with *PGK-lox*P-*EM7-Bsd-bpA* cassette, without MC1-*tk*):
1) pL10 was digested with *Sac*II and *Xho*I, a 4.4 kb fragment was gel purified; 2) pL11 was digested with *Sac*II and *Not*I, a 6.8 kb fragment was gel purified; 3) pWW146 was digested with *Sal*I and *Not*I, a 1.4 kb fragment was gel purified. The three fragments were ligated together in a three-way-ligation to make pWW183.

pL253 is a kind gift from Dr. Pentao Liu. It contains a MC1-*tk* cassette, which can be used for negative selection in mammalian cells.

pWW190 (*E$_2$DH* targeting vector with *PGK*-*lox*P-*EM7*-*Bsd*-*bpA* cassette, with MC1-*tk*):
1) pWW183 was partially digested with *Bam*HI, and then digested with *Sac*II, a 9.6 kb fragment was gel purified; 2) pL253 was partially digested with *Sac*II, and then digested with *Bam*HI, a 5 kb fragment was gel purified. The two fragments were ligated together to make pWW190.

2.1.3 Trapping vectors

2.1.3.1 Promoter trapping vectors

pWW38 (SA-β*geo* cassette):
pSAβ*geo* (Friedrich and Soriano 1991) was cut with *Xho*I, a 4.3 kb fragment was gel purified and cloned into pBS digested with *Xho*I and *Sal*I to make pWW38. The desired orientation of the insert was determined by digestion with *Eco*RI.

pWW62 (*Puro*-*bpA* with multi *lox* sites):
pWW48 was digested with *Bgl*II and *Eco*RI, a 1.2 kb fragment was gel purified and cloned into pBS digested with *Bam*HI and *Eco*RI to make pWW62.

pWW202 (promoter-less *Puro*-*bpA*):
pWW62 was cut with *Hin*dIII and *Xho*I, a 1 kb fragment was gel purified and cloned into pBS digested with *Hin*dIII and *Xho*I to make pWW202.

pWW205 (promoter-less *lox*P-*Puro*-*bpA*):
1) pWW202 was digested with *Hin*dIII and *Xho*I, a 1kb fragment was gel purified; 2) a pair complementary of oligonucleotids, *lox*P-(*Eco*RI-*Hin*dIII)-For and *lox*P-(*Eco*RI-*Hin*dIII)-Rev, were annealed together (Table 2-1); 3) pBS was digested with *Eco*RI and *Xho*I, a 2.9 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way-ligation to make pWW205.

pWW237 (plasmid-based 5' trapping vector):

1) pWW38 was digested with *Bgl*II and *Eco*RI a 4.4 kb fragment was gel purified; 2) pWW205 was digested with *Eco*RI and *Xho*I, a 1 kb fragment was gel purified; 3) pBS was digested with *Bam*HI and *Xho*I, a 2.9 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way-ligation to make pWW237.

pWW239 (5' trapping retrovirus):
1) pWW38 was digested with *Bgl*II and *Eco*RI, a 4.4 kb fragment was gel purified; 2) pWW205 was digested with *Eco*RI and *Xho*I, a 1 kb fragment was gel purified; 3) pMSCV-Neo (Clontech) was digested with *Bam*HI and *Xho*I, a 5.1 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way-ligation to make pWW239.

2.1.3.2 PolyA trapping vectors

CAG promoter and EF1α promoter subcloning vectors are kind gifts from Dr. Haydn Prosser. pVecH1S (regional trapping vector) is a kind gift from Dr. Meredith Wentland. pYTC31 (*PGK-Bsd-bpA*) is a kind gift from Dr. You-Tzung (Bob) Chen.

pWW12 (promoter-less *Bsd-bpA*):
pYTC31 was cut with *Xho*I and *Pst*I, a 0.6 kb fragment was gel purified and cloned into pBS digested with *Xho*I and *Pst*I to make pWW12.

pWW18 (3' trapping retrovirus with *PGK* promoter):
1) pVecH1S was digested with *Hin*dIII, a 1.9 kb fragment was gel purified and digested again with *Bam*HI , a 0.6 kb frament was gel purified; 2) pVecH1S was digested with *Xho*I, a 4.7 kb fragment was gel-purified and digested again with *Hin*dIII, a 4.7 kb fragment was gel purified; 3) pWW12 was digested with *Bam*HI and *Xho*I, a 0.6 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way-ligation to make pWW18.

pWW41 (*CAG* promoter):

CAG promoter subcloning vector was digested with *Hin*cII and *Eco*RI, a 1.6 kb fragment was gel purified and cloned into pBS digested with *Hin*cII and *Eco*RI to make pWW41.

pWW42 (EF1α promoter):
EF1α promoter subcloning vector was digested with *Hin*dIII and *Eco*RI , a 1.3 kb fragment was gel purified and cloned into pBS digested with *Hin*dIII and *Eco*RI to make pWW42.

pWW44 (3' trapping virus with *CAG* promoter, alternative version):
1) pWW18 was digested with *Eco*RI and *Nhe*I, a 4 kb fragment was gel purified; 2) pWW18 was digested with *Eco*RI and *Bgl*II, a 0.7 kb fragment was gel purified; 3) pWW18 was digested with *Nhe*I and *Sal*I, a 0.6 kb fragment was gel purified; 4) pWW41 was digested with *Bam*HI and *Sal*I, a 1.6 kb fragment was gel purified. The four fragments mentioned above were ligated together in a four-way ligation to make pWW44.

pWW45 (3' trapping retrovirus with EF1α promoter, alternative version):
1) pWW18 was digested with *Eco*RI and *Nhe*I, a 4 kb fragment was gel purified; 2) pWW18 was digested with *Eco*RI and *Bgl*II, a 0.7 kb fragment was gel purified; 3) pWW18 was digested with *Nhe*I and *Sal*I, a 0.6 kb fragment was gel purified; 4) pWW42 was digested with *Bam*HI and *Sal*I, a 1.3 kb fragment was gel purified. The four fragments mentioned above were ligated together in a four-way ligation to make pWW45.

pWW59 (PolII-*Neo*-*bpA* with multi *lox* sites):
pWW49 was cut with *Hin*dIII and *Not*I, a 2 kb fragment was gel purified and cloned into pBS digested with *Hin*dIII and *Not*I to make pWW59.

pWW64 (3' trapping virus with *CAG* promoter, final version):
1) pWW44 was partially digested with *Bam*HI, a 7 kb fragment was gel purified and digested again with *Nhe*I and *Not*I, a 5 kb fragment was gel purified; 2) pWW44 was digested with *Xho*I and *Not*I, a 1.5 kb fragment was

gel purified; 3), pWW12 was digested with *Bam*HI and *Xho*I, a 0.5 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way ligation to make pWW64.

pWW65 (3' trapping virus with EF1α promoter, final version):
1) pWW45 was partially digested with *Bam*HI, a 6.7 kb fragment was gel purified and digested again with *Nhe*I and *Not*I, a 4.7 kb fragment was gel purified; 2) pWW45 was digested with *Xho*I and *Not*I, a 1.5 kb fragment was gel purified; 3), pWW12 was digested with *Bam*HI and *Xho*I, a 0.5 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way ligation to make pWW65.

pWW201 (promoter-less *Neo-bpA*):
pWW59 was cut with *Eco*RI and *Xho*I, a 1.1 kb fragment was gel purified and cloned into pBS digested with *Eco*RI and *Xho*I to make pWW201.

pWW238 (plasmid-based 3' trapping vector with *CAG* promoter):
1) pWW64 was digested with *Cla*I and BamHI, a 2.7 kb fragment was gel purified; 2) pWW201 was digested with *Bam*HI and *Xho*I, a 1.1 kb fragment was gel purified; 3) pBS was digested with *Cla*I and *Xho*I, a 2.9 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way ligation to make pWW238.

pWW240 (3' trapping retrovirus with *CAG* promoter in pMSCV backbone):
1) pWW64 was digested with *Cla*I and *Bam*HI, a 2.7 kb fragment was gel purified; 2) pWW201 was digested with *Bam*HI and *Xho*I, a 1.1 kb fragment was gel purified; 3) pMSCV-Neo (Clontech) was digested with *Cla*I and *Xho*I, a 4.1 kb fragment was gel purified. The three fragments mentioned above were ligated together in a three-way ligation to make pWW240.

2.2 Cell culture

2.2.1 ES cell culture condition

ES cell culture was performed as described before (Ramirez-Solis, Davis et al. 1993). Briefly, AB2.2 (129 S7/SvEv$^{\text{Brd-}Hprtb\text{-}m2}$) wild-type ES cells and their

derivatives were always maintained on SNL76/7 feeder cell layers mitotically inactivated treated by γ-irradiation. ES cells were grown in M15 medium (Table 2-2). Cells were cultured at 37 °C with 5% CO_2. If not specified, ES cell medium was changed daily.

When ES cells reached 80-85% confluence, they were ready for passaging. The media was changed about two hours before passaging. After two hours, media was aspirated off, and the plate was washed once with PBS. 2 ml of trypsin was added to each 90-mm plate. The plate was incubated in a TC incubator at 37 °C for 15 minutes. 8 ml of fresh M15 media was added to each well. The cells were dispersed by pipetting up and down vigorously. The ES cell suspension was then evenly distributed to three to four 90-mm feeder plates. The plates were incubated in a TC incubator at 37 °C.

2.2.2 Chemicals used for selection of ES cells

Blasticidin: Blasticidin S HCl (Invitrogen), 1000X stock (5 mg/ml) was made in Phosphate Buffered Saline (PBS). After mixing, the 1000X stock solution was filter sterilized through a 0.2 mm syringe filter.

FIAU: 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracil, 1000X stock (200 μM) was made in PBS and 5 M NaOH was added dropwise until it is dissolved. After mixing, the 1000X stock solution was filter sterilized through a 0.2 mm syringe filter.

G418: Geneticin (Invitrogen), was bought as a sterile stock solution containing 50 mg/ml active ingredient.

Puromycin: ($C_{22}H_{29}N_7O_5$.2HCL, Sigma) 1000X stock (3 mg/ml) was made in MiliQ water. After mixing, the 1000X stock solution was filter sterilized through a 0.2 mm syringe filter.

HAT: 50X HAT supplement (Hypoxanthine-aminopterin-thymidine) (Invitrogen) was bought as a sterile stock solution containing 5 mM Hypoxathine, 20 μM Aminopterin and 0.8 mM Thymidine.

HT: 50X HT supplement (Hypoxanthine-thymidine) (Invitrogen) was bought as a sterile stock solution containing 5 mM Hypoxathine and 0.8 mM Thymidine.

Trypsin: For 5 L, add 35 g NaCl, 5 g D-glucose, 0.9 g $Na_2HPO_4.7H_2O$, 1.85 g KCl, 1.2 g KH_2PO_4, 2 g EDTA, 12.5 g Trpsin (1:250), 15 g Tris base. Adjust the pH from 8.71 to 7.6 with HCl, add phenol to get pink colour. Filter-sterilized and aliquoted into 50 ml falcon tubes, and store at -20 °C.

Table 2.2: Cell culture medium.

Medium Name	Recipe	Purpose
M15	Knockout Dulbecco's Modified Eagle's Medium (DMEM, Gibco/Invitrogen), supplemented with 15% foetal bovine serum (FBS, Gibco/Invitrogen), 2 mM L-glutamine, 50 units/ml penicillin, 40 μg/ml streptomycin and 100 μM β-Mercaptoethanol (β-ME)	Culture of undifferentiated ES cells
M10	Knockout Dulbecco's Modified Eagle's Medium (DMEM, Gibco/Invitrogen) supplemented with 10% foetal bovine serum (FBS, Gibco/Invitrogen), 2 mM L-glutamine, 50 U/ml penicillin, and 40 μg/ml streptomycin	Culture of feeder cells and Phoenix cells
Viral Production Medium	M10 medium supplemented with heat-inactivated FBS	Virus production
Differentiation Medium	For 100 ml Knockout Dulbecco's Modified Eagle's Medium (DMEM, Gibco/Invitrogen), 25 ml FBS (Gibco/Invitrogen), 1.25 ml 200 mM 100X L-glutamine stock (Gibco/Invitrogen), 1.25 ml 10mM β-ME stock (10mM) and 1.25 ml 100X nonessential amino acids (NEAA) stock (Gibco/Invitrogen) were added	ES cell *in vitro* differentiation

2.2.3 Transfection of DNA into ES cells by electroporation

DNA used for ES cell transfection was normally prepared using a Qiagen Plasmid Purification Kit (Qiagen). If DNA was used for gene targeting, it would be linearized by digestion with an appropriate enzyme under the conditions recommended by the manufacturers. If DNA was used for transient expression, the linearization step would be omitted. Before electroporation, DNA was purified by ethanol precipitation and air-dried briefly in a tissue culture (TC) hood. The air-dried DNA was then dissolved in sterile 1X TE buffer (pH 8.0) to a final concentration of about 1 μg/μl. Unless specified, 20 μg DNA was used for each electroporation.

ES cell electroporation was performed according to standard protocols (Ramirez-Solis, Davis et al. 1993). Briefly, ES cells (80% confluent) were fed 2-3 hours before harvesting. Immediately before electroporation, ES cells were trypsinized and resuspend in M15 media. The cells were collected by centrifuging and washed once in PBS. The cells were resuspended in PBS to a final concentration of $1X10^7$ cells/ml. $1X10^7$ ES cells were transferred into a 0.4 cm gap curvette (Biorad) together with 20 μg DNA. The electroporation was carried out using a Biorad “Gene Pulser” at 230 V, 500 μF. After electroporation, ES cells were plated onto a 90-mm feeder plate and unless stated otherwise, were cultured for 10 days to allow the formation of single ES cell colonies. Drug selection was usually initiated 24 hours post-electroporation.

2.2.4 Picking ES cell colonies

50 μl of trypsin was added to each well of a 96-well round bottom plate by using a multi-channel pipette. After washing a 90-mm tissue culture plate for picking with PBS, about 8 ml PBS was added to cover the plate. The colonies were picked from the 90-mm plate by using a P20 Pipetman set at 10 μl and transferred into the wells with trypsin. After completing a 96-well plate, the plate was incubated in a TC incubator at 37 °C for 10 to 15 minutes. After that, 150 μl of fresh M15 media was added to each well. The colonies were

broken up by pipetting up and down vigorously. The ES cell suspension was then transferred to a 96-well feeder plate. The plate was incubated in a TC incubator at 37 °C.

2.2.5 Passaging ES cells

When ES cells in most wells on a 96-well plate reached 80-85% confluence (determined both by the change of the medium colour and by checking the plate under a microscope), the plate was judged ready for passaging. The media was changed about two hours before passaging. After two hours, media was aspirated off, and the plate was washed once with PBS. 50 µl of trypsin was added to each well of a 96-well plate by using a multi-channel pipette. The plate was incubated in a TC incubator at 37 °C for 15 minutes. 150 µl of fresh M15 media was added to each well. The cells were separated by pipetting up and down vigorously. The ES cell suspension was then evenly distributed to three to four 96-well feeder/gelatinized plates. The plates were incubated in a TC incubator at 37 °C.

2.2.6 Freezing ES cells

When ES cells reached 80-85% confluence, they were ready for freezing. The media was changed about two hours before passaging. After two hours, media was aspirated off, and the plate was washed once with PBS. 50 µl of trypsin was added to each well of a 96-well plate by using a multi-channel pipette. The plate was incubated in a TC incubator at 37 °C for 15 minutes. 50 µl of 2X Freezing Media (60% DMEM, 20% FCS, 20% DMSO) was added to each of the wells and the cells were broken up by pipetting up-and-down. 100 µl of filter-sterilized (0.22 um) Mineral Oil was added to each well. The plate was put into a polystyrene box with lid and frozen at –80 °C.

2.2.7 Thawing ES cells

To thaw frozen ES cell clones, the 96-well plate was taken out of the -80 °C freezer and placed immediately into the 37 °C incubator. After all of the wells thawed completely, the clones were transferred to appropriately labelled wells in 24-well feeder plates pre-equilibrated with 2 ml of M15 media per well. For

maximum recovery of sample, another 200 µl of M15 was added to rinse each well and the cell suspension was transferred to the appropriate wells in the 24-well feeder plates. The plates were incubated in a TC incubator at 37 °C.

2.2.8 Cre-mediated recombination to pop out the selection cassettes

20 µg of the Cre expression plasmid pCAAG-Cre (Araki, Araki et al. 1995) was electroporated into $1X10^7$ ES cells. After electroporation, the cells were serially diluted in M15 and about 1,000 ES cells were plated onto a 90-mm feeder plate and cultured for 10 days to allow the formation of single ES cell colonies. 96 ES cell clones were picked into a 96-well feeder plate. To identify clones with Cre-mediated recombination events, the 96 well plates were replicated. Sib-selection was performed to identify ES clones with correct drug resistance pattern. The right clones were expanded and confirmed by Southern analysis.

2.2.9 Generation of targeted ES cell lines

WW14 (AB2.2 targeted with pWW74):

20 µg of pWW74 was linearized with *Sca*I and electroporated into AB2.2 cells (#239, passage 17). The transfectants were selected with puromycin and FIAU simultan*eo*usly for 8 days. 96 puromycin resistant clones were picked and expanded on a 96-well feeder plate. Genomic DNA was extracted and digested with *Bam*HI for Southern analysis using a *D11Mit71* 3' probe to identify gene-targeting events. The expected sizes of the detected restriction fragments were 14.1 kb for wild-type allele and 10.3 kb for the targeted allele. The correctly targeted clones were expanded and named WW14.

WW16 (AB2.2 targeted with pWW75):

20 µg of pWW75 was linearized with *Sca*I and electroporated into AB2.2 cells (#239, passage 17). The transfectants were selected with G418 and FIAU simultaneously for 8 days. 96 G418 resistant clones from each cell line were picked and expanded on a 96-well feeder plate. Genomic DNA was extracted and digested with *Bam*HI for Southern analysis using a *D11Mit71* 3' probe to identify gene-targeting events. The expected sizes of the detected restriction

fragments were 14.1 kb for the wild-type allele and 17.9 kb for the targeted allele. The correctly targeted clones were expanded and named WW16.

WW24 (WW14 targeted with pWW75):
20 μg of pWW75 was linearized with *Sca*I and electroporated into WW14. The transfectants were selected with G418 and FIAU simultaneously for 8 days. 96 G418 resistant clones were picked and expanded on a 96-well feeder plate. Genomic DNA was extracted and digested with *Bam*HI for Southern analysis using a *D11Mit71* 3' probe to detect gene-targeting events. The double-targeted clones would have a 10.3 kb restriction fragment (3' *Hprt* targeting) and a 17.9 kb restriction fragment (5' *Hprt* targeting). The correctly targeted clones were expanded and named WW24. The function of the 5' *Hprt* and 3' *Hprt* cassettes were tested by transient Cre expression and subsequent HAT selection.

WW25 (WW16 targeted with pWW74):
20 μg of pWW74 was linearized with *Sca*I and electroporated into WW16. The transfectants were selected with puromycin and FIAU simultaneously for 8 days. 96 puromycin resistant clones were picked and expanded on a 96-well feeder plate. Genomic DNA was extracted and digested with *Bam*HI for Southern analysis using a *D11Mit71* 3' probe to detect gene-targeting events. The double-targeted clones would have a 10.3 kb restriction fragment (3' *Hprt* targeting) and a 17.9 kb restriction fragment (5' *Hprt* targeting). The correctly targeted clones were expanded and named WW25. The function of the 5' *Hprt* and 3' *Hprt* cassettes were tested by transient Cre expression and subsequent HAT selection.

Table 2.3: ES Cell lines constructed for the project.

ES Cell Line	Targeted Locus	Starting Cell line	Targeting Vector / Cre expression plasimid	Linearization Enzyme	Drug Selection	Genomic DNA digestion	Probe and sizes of fragments	Drug Resistance and Sensitivity
WW14	*D11Mit71*	AB2.2	pWW74	*Sca* I	puromycin+FIAU	*Bam* HI (*D11Mit71* 5' probe), *Xba* I (*D11Mit71* 3' probe)	*D11Mit71* 5' probe: 7.1 kb (W.T.), 4.9 kb (3' *Hprt*). *D11Mit71* 3' probe: 14.1 kb (W.T.), 10.3 kb (3' *Hprt*).	Neo^S, $Puro^R$, Bsd^S, HAT^S
WW16	*D11Mit71*	AB2.2	pWW75	*Sca* I	G418+FIAU	*Bam* HI (*D11Mit71* 5' probe), *Xba* I (*D11Mit71* 3' probe)	*D11Mit71* 5' probe: 7.1 kb (W.T.), 6.4 kb (5' *Hprt*). *D11Mit71* 3' probe: 14.1 kb (W.T.), 17.9 kb (5' *Hprt*).	Neo^R, $Puro^S$, Bsd^S, HAT^S
WW24	*D11Mit71*	WW14-B2	pWW75	*Sca* I	G418+FIAU	*Bam* HI (*D11Mit71* 5' probe), *Xba* I (*D11Mit71* 3' probe)	*D11Mit71* 5' probe: 6.4 kb (5' *Hprt*), 4.9 kb (3' *Hprt*). *D11Mit71* 3' probe: 17.9 kb (5' *Hprt*), 10.3 kb (3' *Hprt*).	Neo^R, $Puro^R$, Bsd^S, HAT^S
WW25	*D11Mit71*	WW16-B2	pWW74	*Sca* I	puromycin+FIAU	*Bam* HI (*D11Mit71* 5' probe), *Xba* I (*D11Mit71* 3' probe)	*D11Mit71* 5' probe: 6.4 kb (5' *Hprt*), 4.9 kb (3' *Hprt*). *D11Mit71* 3' probe: 17.9 kb (5' *Hprt*), 10.3 kb (3' *Hprt*).	Neo^R, $Puro^R$, Bsd^S, HAT^S
WW45	*D11Mit71*	WW24-A1	pCCAG-Cre	N.A.	No selection	*Xba* I (*D11Mit71* 5' probe)	*D11Mit71* 5' probe: 6.4 kb (5' *Hprt*), 4.9 kb (3' *Hprt*).	Neo^S, $Puro^S$, Bsd^S, HAT^S
WW46	*D11Mit71*	WW25-C1	pCCAG-Cre	N.A.	No selection	*Xba* I (*D11Mit71* 5' probe)	*D11Mit71* 5' probe: 6.4 kb (5' *Hprt*), 4.9 kb (3' *Hprt*).	Neo^S, $Puro^S$, Bsd^S, HAT^S
WW69	*E_2DH*	WW45-B2	pWW190	*Sac* II	blasticidin+FIAU	*Eco* RI (*E_2DH* 5' probe), *Nde* I (*E_2DH* 3' probe)	*E_2DH* 5' probe: 14.9 kb (W.T.), 9.2 kb (targeted). *E_2DH* 3' probe: 13.1 kb (W.T.), 9.6 kb (targeted).	Neo^S, $Puro^S$, Bsd^R, HAT^S
WW93	*E_2DH*	WW69-D6	pCCAG-Cre	N.A.	HAT/HT	*Eco* RI (*E_2DH* 5' probe), *Nde* I (*E_2DH* 3' probe)	*E_2DH* 5' probe: 9.2 kb (targeted). *E_2DH* 3' probe: 9.6 kb (targeted).	Neo^S, $Puro^S$, Bsd^R, HAT^R
WW99	Random retroviral insertion	WW69-D6	pWW239 derived gene trap virus	N.A.	G418	*Kpn* I (*LacZ* probe)	*LacZ* probe: 6.0/6.9 kb (proviral insertion).	Neo^R, $Puro^S$, Bsd^R, HAT^S
WW103-RT	Cre induced inversion	WW99	pCCAG-Cre	N.A.	puromycin	*Kpn* I (*LacZ* probe)	*LacZ* probe: 19.0 kb (inversion).	Neo^R, $Puro^R$, Bsd^R, HAT^S
WW103	Cre induced mitotic recombination	WW103-RT	pCCAG-Cre	N.A.	HAT/HT	*Nde* I (*E_2DH* 3' probe), *Eco* RI & *Spe* I (*LacZ* probe)	*E_2DH* 3' probe: 9.6 kb (targeted). LacZ probe: restriction fragments of various lengths (proviral/host junction fragment).	Neo^R, $Puro^R$, Bsd^R, HAT^R
WW100	Random vector insertion	WW69-D6	pWW237	*Sca* I	G418	N.A.	N.A.	Neo^R, $Puro^S$, Bsd^R, HAT^S
WW104	Cre induced inversion	WW100	pCCAG-Cre	N.A.	puromycin	N.A.	N.A.	Neo^R, $Puro^R$, Bsd^R, HAT^S
WW106	Cre induced mitotic recombination	WW104	pCCAG-Cre	N.A.	HAT/HT	*Nde* I (*E_2DH* 3' probe), *Eco* RI & *Spe* I (*LacZ* probe)	*E_2DH* 3' probe: 9.6 kb (targeted). LacZ probe: restriction fragments of various lengths (vector/host junction fragment)	Neo^R, $Puro^R$, Bsd^R, HAT^R

WW45 (WW24 selection markers pop-out):
20 μg of supercoiled pCAAG-Cre was electroporated into WW24. About 1,000 ES cells were plated onto a 90-mm feeder plate and cultured for 10 days to allow the formation of single ES cell colonies. 96 clones were picked and expanded on a 96-well feeder plate. The 96-well plate was replicated and sib-selection was performed to identify ES clones in which both *Neo* and *Puro* cassettes were popped out, but no recombination had happened between the two half *Hprt* cassettes. The correct recombinants should be G418 sensitive, puromycin sensitive and HAT sensitive. The clones showing this pattern of sensitivity were expanded and confirmed by Southern analysis using a *D11Mit71* 3' probe. The double-targeted clones would have a 10.3 kb *Bam*HI restriction fragment (3' *Hprt* targeting) and a 15.9 kb *Bam*HI restriction fragment (5' *Hprt* targeting and *Neo* pop-out). The function of the 5' *Hprt* and 3' *Hprt* cassettes were tested by transient Cre expression and subsequent HAT selection.

WW46 (WW25 selection markers pop-out):
20 μg of supercoiled pCAAG-Cre was electroporated into WW25. About 1,000 ES cells were plated onto a 90-mm feeder plate and cultured for 10 days to allow the formation of single ES cell colonies. 96 clones were picked and expanded on a 96-well feeder plate. The 96-well plate was replicated and sib-selection was performed to identify ES clones in which both *Neo* and *Puro* cassettes were popped out, but no recombination had happened between the two half *Hprt* cassettes. The correct recombinants should be G418 sensitive, puromycin sensitive and HAT sensitive. The clones showing this pattern of sensitivity were expanded and confirmed by Southern analysis using a *D11Mit71* 3' probe. The double-targeted clones would have a 10.3 kb *Bam*HI restriction fragment (3' *Hprt* targeting) and a 15.9 kb *Bam*HI restriction fragment (5' *Hprt* targeting and *Neo* pop-out). The function of the 5' *Hprt* and 3' *Hprt* cassettes were tested by transient Cre expression and subsequent HAT selection.

WW69 (WW45 targeted with pWW190):
20 μg of pWW190 was linearized with *Sca*I and electroporated into WW45. The transfectants were selected with blasticidin and FIAU simultaneously for 8 days. 96 blasticidin resistant clones were picked and expanded on a 96-well feeder plate. Genomic DNA was extracted and Southern analysis was performed using several different probes to determine the genotype of the clones: 1) *Bam*HI digestion, hybridized with a *D11Mit71* 3' probe, the correct clones would have a 10.3 kb restriction fragment (3' *Hprt* targeting) and a 15.9 kb restriction fragment (5' *Hprt* targeting and *Neo* pop-out). 2) *Xba*I digestion, hybridized with a *D11Mit71* 5' probe (pWW116), the correctly targeted clones would have a 6.4 kb restriction fragment (5' *Hprt* targeting and *Neo* pop-out) and a 5.0 kb restriction fragment (3' *Hprt* targeting and *Puro* pop out); 3) *Eco*RI digestion, hybridized with an E_2DH 5' probe (pL16, (Liu, Zhang et al. 1998)), the targeted restriction fragment is 9.2 kb and the wild-type restriction fragment is 14.9 kb; 4) *Nde*I digestion, hybridized with an E_2DH 3' probe (pL17, (Liu, Zhang et al. 1998)), the targeted restriction fragment is 9.6 kb and the wild-type restriction fragment is 13.1 kb. Two correctly targeted clones were identified by Southern analysis, WW69-C8 and WW69-D6.

To use induced mitotic recombination to make the homozygous mutations, the E_2DH end point targeting cassette needs to be on the same chromosome as the 3' *Hprt* cassette. To determine the location of the *PGK-lox*P-*Bsd-bpA* cassette, the WW69-C8 and D6 clones were expanded and supercoiled pCAAG-Cre was electroporated into both. The recombinants were selected with HAT for 6 days and HT for another 4 days. 36 HAT resistant clones were picked from each electroporation and cultured on a 96-well feeder plate. Genomic DNA was extracted and Southern analysis was performed using the *D11Mit71* 5' and 3' probe as well as the E_2DH 5' and 3' probe. The cell line WW69-D6 was determined to have both the right genotype and *PGK-lox*P-*Bsd-bpA* cassette location. Single cell subclones were isolated to avoid possible contamination of other cells. The subclones were confirmed by Southern and sib-selection. The correct recombinants should be G418 sensitive, puromycin sensitive, HAT sensitive and blasticidin resistant.

WW93 (WW69-D6 induced mitotic recombination):
20 μg of supercoiled pCAAG-Cre was electroporated into WW69-D6. The recombinants were selected with HAT for 6 days and HT for another 4 days. 96 clones were picked and expanded on a 96-well feeder plate. The plate was replicated and sib-selection was performed to determine the drug resistance of the clones. The correct recombinants should be G418 sensitive, puromycin sensitive, HAT resistant and blasticidin resistant. The clones with this combination of drug resistance and sensitivity were expanded and confirmed by Southern analysis using the *E_2DH* 5' and 3' probe: 1) *Eco*RI digestion, hybridized with an *E_2DH* 5' probe. The correct recombinants would only have the 9.2 kb targeted restriction fragment but not the 14.9 kb wild-type restriction fragment; 2) *Nde*I digestion, hybridized with *E_2DH* 3' probe, The correct recombinants would only have the 9.6 kb targeted restriction fragment but not the 13.1 kb wild-type restriction fragment. One of the correct clones, WW93-A12 was expanded and single cell subcloned to avoid possible contamination by other cells. This cell line was used as control for the ES cell *in vitro* differentiation.

2.2.10 Retroviral approaches

2.2.10.1 Retrovirus production

The Phoenix ecotropic retroviral packaging cell line (Grignani, Kinsella et al. 1998), a derivative of human embryonic kidney 293T line expressing retroviral *gal*, *pol* and *env* proteins, was obtained from the American Tissue Culture Collection (Manassas, Virginia, USA). Cells were cultured according to the protocols on Dr. Garry Nolan's lab webpage (http://www.stanford.edu/group/nolan). Briefly, the Phoenix cells were cultured in M10 medium (Table 2-2) at 37°C with 5% CO_2. The medium was changed every 2-3 days. Cells were split 1:5 when they reached 70-80% confluence.

24 hours prior to transfection, Phoenix cells were plated at a density of 2X 10^6 cells per 90-mm plate in M10. 2-3 hours before transfection, cells were fed with 14 ml fresh M10 medium (at this time the cells were about 60% confluent).

CalPhos™ Mammalian Transfection Kit (BD Bioscience) was used for transient transfection of the Phenoix retroviral packaging cell line. Briefly, DNA prepared with the Qiagen Plasmid Purification Kit (Qiagen) was precipitated with ethanol, air-dried and then dissolved in appropriate volume of TE. For each transfection of cells on each 90-mm plate, 25 μg DNA was mixed with 86.8 μl 2 M Calcium Phosphate Solution. Sterile water was added to make a final volume of 700 μl. The calcium solution containing DNA was added dropwise to 700 μl 2X HEPES-buffered Saline (HBS) solution, while being mixed quickly by bubbling vigorously with a 1 ml sterile pipette and an autopipettor.

The DNA mixture was incubated at room temperature for 20 minutes, vortexed gently and then added dropwise to the culture plate medium. 24 hours after transfection, the calcium phosphate-containing medium was removed, plates were washed twice with PBS and 10 ml of fresh Viral Production Medium (Table 2-2) was added to each plate. Viral supernatant was harvested 36, 48, 60 and 72 hours after transfection and stored immediately in a –80°C freezer.

2.2.10.2 Viral Infection

ES cells were plated at a density of 3X 10^6 cells per 90-mm feeder plate about 24 hours before infection. The viral supernatant collected from all the time points was mixed together and filtered through a 0.45 μm filter. Heat-inactivated FBS was added to the viral supernatant to make the final concentration of FBS up to 15%. Polybrene (Hexadimethrine Bromide, Sigma) was added to the viral supernatant to a final concentration of 4 μg/ml. 12 ml viral supernatant was added to each plate of ES cells. The viral supernatant was replaced every 12 hours with fresh supernatant. After 48 hours of infection, the viral supernatant was removed and fresh M15 medium was added. The drug selection was applied 24 hours after infection was stopped.

2.2.10.3 Titration of the retrovirus

ES cells were plated at a density of 3X 10^6 cells per 90-mm feeder plate. 24 hours later, 1 ml or 10 ml of viral supernatant was applied to each plate. For the virus carrying a *Neo* cassette, G418 selection (180 μg/ml) was initiated 24 hours after viral infection and continued for 8 days. The drug-resistant ES colonies were stained with 2% methylene blue in 70% ethanol and counted. The titre of the retrovirus is defined as the number of drug resistant ES cell colonies per milliliter of viral supernatant used to infect the cells.

2.2.11 Gene trap mutagenesis using the retroviral vector

2.2.11.1 Gene trapping

WW99 (WW69-D6 infected with pWW239-derived retrovirus):
Gene-trap retrovirus was produced by transient transfection of Phoenix viral packaging cells. A total of 2000 ml of viral supernatant was harvested and filtered through 0.45 μm filters. WW69-D6 ES cells were plated on a total of twenty 90-mm feeder plates at a density of 3X 10^6 cells per plate (WW99-1 to WW90-20). 24 hours later, each plate of cells was infected with 12 ml of viral supernatant. Viral supernatant was replaced by fresh supernatant every 12 hours. After 48 hours, the viral supernatant was removed and fresh M15 medium was added to each plate. G418 selection (180 μg/ml) was initiated 24 hours after the viral infection terminated. Drug selection was continued for 10 days until the G418 resistant colonies were clearly visible. One plate (WW99-20) was stained with 2% methylene blue in 70% ethanol to determine the number of gene-trap clones obtained. The G418 resistant ES cell colonies from each of the remaining 19 retrovirus infected plates were separately trypsinized, resuspended in M15 medium and plated as a pool onto 19 feeder plates (WW99-1 to WW99-19). These cells were selected with G418 until they reached about 80% confluence. 1X10^7 cells were used for the Cre-mediated inversions. The rest of the cells were frozen down for the stock.

2.2.11.2 Cre-mediate inversion

WW103-RT (WW99 regional trapping):

20 μg of supercoiled pCAAG-Cre was electroporated into $1X10^7$ cells from the WW99-1 to WW99-19 pools. Puromycin selection (3 μg/ml) was initiated 24 hours after the electroporation. The drug selection was continued for 6 days until the colonies were visible under microscope. Selection was then released, and the colonies were grown in M15 medium for another 4 days. The puromycin resistant ES cell colonies from each of the 19 plates were trypsinized, resuspended in M15 medium and maintained as 19 separate pools on 19 feeder plates (WW103-RT-1 to WW103-RT-19). These cells were selected with puromycin until they reached about 80% confluence. $1X10^7$ cells were used for the Cre-induced mitotic recombination. The rest of the cells were frozen down for the stock.

2.2.11.3 Cre-induced mitotic recombination

WW103 (WW103-RT induced mitotic recombination):

20 μg of supercoiled pCAAG-Cre was electroporated into $1X10^7$ cells of WW103-RT-1 to WW103-RT-19 pools. HAT selection was initiated 24 hours after the electroporation. The drug selection continued for 6 days until the colonies were visible under microscope and the colonies were grown in M15 medium with HT supplement for another 4 days. 48 HAT resistant ES cell colonies from each of the 19 plates were picked and expanded on 96-well feeder plate.

All of the 96-well plates were replicated and sib-selection was performed to determine the drug resistance of the clones. Cells on a 96-well feeder plate were split 1:5 onto 5X gelatinized 96-well tissue culture plates. These five plates were selected with M15, M15+G418, M15+puromycin, M15+HAT, and M15+blasticidin, respectively. Once most drug resistant clones on the plates grew to about 100% confluence, these plates were stained with 2% methylene blue in 70% ethanol, and drug resistance of each clone was scored. The correct recombinants should be G418 resistant, puromycin resistant, HAT resistant and blasticidin sensitive.

Genomic DNA was extracted and Southern analysis was performed using the *E_2DH* 3' probe and a *lacZ* probe (a 800 kb *Bam*HI-*Cla*I fragment from pWW239): 1) *Nde*I digestion, hybridized with the *E_2DH* 3' probe, the correct recombinants would only have the 9.6 kb targeted restriction fragment but no 13.1 kb wild-type restriction fragment; 2) *Eco*RI digestion, hybridized with the *lacZ* probe; 3) *Spe*I digestion, hybridized with the *lacZ* probe. All clones that are homozygous for the *E_2DH* locus presumably also carry homozygous mutations at the trapped locus. Individual trapping events were identified by their unique proviral/host junction generated by two different restriction enzyme digestions (*Eco*RI and *Spe*I)

All the homozygous clones from the 19 plates were grouped according to the sizes of their proviral junction fragments. For the groups that have more than one clone, at least 2 independent clones were expanded. For the groups that only have one clone, the clone was expanded. Genomic DNA and RNA were extracted from all the expanded clones. Southern analysis was carried out using different probes and enzyme digestions to confirm the clones and determine their genotypes: 1) *Eco*RI digestion, hybridized with the *E_2DH* 5' probe, the correct recombinants would only have the 9.2 kb targeted restriction fragment but not the 14.9 kb wild-type restriction fragment; 2) *Nde*I digestion, hybridized with the *E_2DH* 3' probe, the correct recombinants would only have the 9.6 kb targeted restriction fragment but not the 13.1 kb wild-type restriction fragment; 3) *Eco*RI digestion, hybridized with the *lacZ* probe; 4) *Spe*I digestion, hybridized with the *lacZ* probe; 5) *Kpn*I digestion, hybridized with the *lacZ* probe. The *Kpn*I digestion and hybridization using the *lacZ* probe was used to determine whether the clones carry homozygous inversions.

2.2.12 Gene trap mutagenesis using plasmid based vector

WW100 (WW69-D6 cells electroporated with pWW237):
20 μg *Sca*I linearized pWW237 DNA was electroporated into $1X10^7$ WW69-D6 ES cells. Ten electroporations were carried out and the cells were plated on ten 90-mm feeder plates. G418 selection (180 μg/ml) was initiated 24

hours after electroporation. The drug selection continued for 10 days when the G418 resistant colonies were clearly visible. The G418 resistant ES cell colonies from each of the 10 plates were trypsinized, resuspended in M15 medium and maintained as separate pools on 10X 90-mm feeder plates (WW100-1 to WW100-10). These cells were selected with G418 until they reached about 80% confluence. $1X10^7$ cells were used for the Cre-mediated inversions. The rest of the cells were frozen down for the stock.

WW104 (WW100 regional trapping):
20 μg of supercoiled pCAAG-Cre was electroporated into $1X10^7$ cells of WW100-1 to WW100-10 pools. Puromycin selection (3 μg/ml) was initiated 24 hours after the electroporation. The drug selection continued for 6 days until the colonies were visible under microscope and the colonies were transferred into M15 medium for another 4 days. The puromycin resistant ES cell colonies from each of the 10 plates were trypsinized, resuspended in M15 medium and maintained as 10 separate pools on 10X 90-mm feeder plates (WW104-1 to WW104-10). These cells were selected with puromycin until they reached about 80% confluence. $1X10^7$ cells were used for the Cre-induced mitotic recombination. The rest of the cells were frozen down for the stock.

WW106 (WW104 induced mitotic recombination):
20 μg of supercoiled pCAAG-Cre was electroporated into $1X10^7$ cells of the pools WW104-1 to WW104-10. HAT selection was initiated 24 hours after the electroporation. The drug selection continued for 6 days until the colonies were visible under microscope and the colonies were transferred into M15 medium with HT supplement for another 4 days. 48 HAT resistant ES cell colonies from each of the 19 plates were picked and expanded on 96-well feeder plate.

Sib-selection and Southern analysis were carried out in essentially the same way as the gene-trap mutagenesis using the retrovirus.

2.2.13 ES cell *in vitro* differentiation

Embryoid bodies were established and cultured as described before (Wobus, Guan et al. 2002). In brief, ES cells were grown on 90-mm or 6-well feeder plates until they reached 70-80% confluence. The cells were fed 2-3 hours before trypsinization. The plates were washed in PBS and trypsinized for 15 minutes. The cells were resuspended in M15 and counted using a Coulter Counter (Beckman). The cells were diluted in Differentiation Medium (Table 2-2) to a final concentration of 600 cells per 20 μl. 20 μl drops of ES cell suspension was placed on the bottom of 100-mm bacteriological Petri dishes. The bacteriological dishes were inverted (upside down) and the hanging drops of ES cell aggregates cultured at 37°C with 5% CO_2.

After two days (Day 2), 15 ml Differentiation Medium was put into each bacteriological dishes, and the aggregates were rinsed off the bottom into the media. The aggregates were cultured in suspension at 37°C with 5% CO_2. After another three days (day 5), the EBs from each dish were transferred into a 15-ml falcon tube. The EBs sedimented by gravity and the medium was discarded and replaced with Differentiation Medium supplemented with 10^{-8} M RA. The EBs were resuspended by inverting for several times and transferred to gelatinized 90-mm tissue culture plates. One plate of EBs were washed in PBS and used to extract RNA at day 5. The culture medium was changed every other day during the differentiation process. RNA samples were taken at various time points.

2.3 DNA methods

2.3.1 Probes

LacZ probe: A probe for gene-trap viruses containing the SA*βgeo* gene-trap cassette, consisting of a 1.4 kb *Cla*I fragment from pSA*βgeo,* a plasmid containing the *SAβgeo* cassette in pBS (from Dr. Philippe Soriano).

Neo probe: A probe for gene-trap viruses containing the SAβgeo gene-trap cassette and consisting of a 700 bp *Pst*I /*Xba*I fragment from the *PGK-Neo* cassette.

E$_2$DH 5' probe: A 2.1 kb *Nhe*I-*Not*I genomic fragment was cloned into *Xba*I-*Not*I digested pBS vector to make pL16 (Liu, Zhang et al. 1998). pL16 was cut with *Bgl*II and *Not*I, and an 1.7 kb fragment was gel purified to be used as *E$_2$DH* 5' probe.

E$_2$DH 3' probe: An 1.7 kb *Spe*I-*Sac*I genomic fragment was cloned into *Xba*I-*Sac*I digested pBS vector to make pL17 (Liu, Zhang et al. 1998). pL17 was cut with *Eco*RI and *Sac*I, and an 1.7 kb fragment was gel purified to be used as *E$_2$DH* 3' probe.

D11Mit71 3' probe: pBZ84 was isolated from a 3' *Hprt* lirary using a pair of *D11Mit71* specific primers (Zheng, Sage et al. 2000). pBZ84 was cut with *Asc*I and *Xho*I, and a 3.6 kb fragment was gel purified to be used as *D11Mit71* 3' probe.

Genomic probes:
Genomic DNA probes were PCR amplified from AB2.2 mouse genomic DNA and used for Southern-blot analysis. PCR products were routinely cloned into TOPO TA Cloning Vector (Invitrogen). The probes were made by digestion of the plasmid DNA using appropriate enzymes and gel purified. The concentration of the probe DNA was determined by spectrophotometer (Beckman) and/or gel electrophoresis.

D11Mit71 5' probe:
D11Mit71-5' probe-F
5'-CCC TAA CCA GGA TAG ATA CTG CTT GCT TTG TG-3'
D11Mit71-5' probe-R
5'-GCT TGG GGG TCA CTA CAA CTT GAA GAA CTG-3'

Pecam trapping probe:
Pecam-trapping-F
5'-CTG GCA CCT TTC TCC AGT GAA CCG TCC-3'
Pecam-trapping-R

5'-CCT CTG GCA TCA AGG AGG TCT TGG TCT G-3'

Acly 5' trapping probe:
Acly-5' Probe-F
GCTGCGTCAAGGAGTGGAGACCTATGG
Acly-5' Probe-R
GGCTGGGTACTGAA*CAG*TGTCCT*CAG*G

Acly 3' trapping probe:
Acly-3' Probe-F
GGCCTGACCTGGGGCTGATGGG
Acly-3' Probe-R
GGTACCTGTTAGACTGGGCGCTC*CAG*

2.3.2 Southern blotting and hybridization

2.3.2.1 Southern blotting

2-5 μg genomic DNA was digested with an appropriate restriction enzyme overnight. The digested fragments were separated by electrophoresis on 0.8% agarose gel. After electrophoresis, the gel was first soaked in Depurination Buffer (0.25 M HCl) for 10 minutes with gentle agitation, and then transferred into Denaturation Buffer (0.5 M NaOH, 1.5 M NaCl) for 1 hour with gentle agitation. A capillary blot was set up according to standard methods. Denaturation Buffer was used as the transfer buffer. Following overnight transfer, the blot was neutralized in Membrane Rinse Buffer (0.2 M Tris-Cl (pH7.4), 2X SSC) for 5 minutes, and baked at 80 °C for 1 hour.

2.3.2.2 Probe preparation

Probe DNA was labelled using Rediprime™ II Random Prime Labeling System (Amersham) according to the manufacturer's instructions. Briefly, 20 ng DNA was diluted in a final volume of 45 μl 1X TE buffer. The DNA sample was denatured by heating to 100 °C for 5 minutes and then placed on ice for another 5 minutes. The denatured DNA was added to a reaction tube. 5 μl Redivue [^{32}P] dCTP was added and the labelling solution was mixed

thoroughly by pippetting up and down. The tube was incubated at 37 °C for 10-30 minutes and the purified with a pre-filled G-50 column. The purified probe was denatured at 100 °C for 5 minutes, and chilled on ice for another 5 minutes before use.

2.3.2.3 Hybridization

Blots were pre-hybridized at 65 °C for at least one hour in Hybridization Buffer (1.5X SSCP, 1X Denhardts solution, 0.5% SDS, 10% Dextran Sulfate) supplemented with denatured herring sperm DNA. After pre-hybridization, the denatured probe was added and the blot was hybridized at 65°C overnight. The next day, the blot was first rinsed briefly in low stringency wash buffer (1X SSC, 0.1% SDS) at room temperature and then washed in high stringency wash buffer (0.5X SSC and 0.1% SDS) at 65 °C for 15 minutes. The blot was then exposed to X-ray film (Fuji).

2.3.3 Splinkerette PCR

2.3.3.1 Splinkerette adaptors preparation

Splinkerette PCR was carried out as described previously (Mikkers, Allen et al. 2002). 150 pmol of HMSpAa, 150 pmol of HMSpBb and 5 μl NEB Buffer 2 (New England Biolabs) were used to make a 100 μl oligonucleotide mixture. The mixture was denatured by heating to 95 °C for 3 minutes, and then annealed by slowly cooling to room temperature.

Splinkerette Oligos:
HMSpAa: 5'-CGA AGA GTA ACC GTT GCT AGG AGA GAC CGT GGC TGA ATG AGA CTG GTG TCG ACA CTA GTG G-3'
HMSpBb-*Sau*3AI
5'-gatc CCA CTA GTG TCG ACA CCA GTC TCT AAT TTT TTT TTT CAA AAA AA-3'
HMSpBb –*Xba*I
5'-ctag CCA CTA GTG TCG ACA CCA GTC TCT AAT TTT TTT TTT CAA AAA AA-3'
HMSpBb -*Eco*RI

5’-aatt CCA CTA GTG TCG ACA CCA GTC TCT AAT TTT TTT TTT CAA AAA AA-3’

2.3.3.2 Genomic DNA digestion and ligation with Splinkerette adaptors

2 μg of genomic DNA was digested with *Sau*3AI in a 30 μl volume at 37 °C for 3 hours. The *Sau*3AI enzyme was heat-inactivated by incubating at 65 °C for 20 minutes. For a 20 μl ligation mixture, 3 μl of the annealed Splinkerette adaptors, 5 μl digested genomic DNA, 2 μl 10X Ligation Buffer and 5 units T4 DNA Ligase (New England Biolabs) were added. The ligation mixture was incubated at 16 °C overnight. The T4 DNA ligase was heat-inactivated by incubating at 65 °C for 15 minutes. The ligation product was then digested with *Cla*I. For a 20 μl *Cla* I digestion mixture, 10 units *Cla*I, 4 μl 10X NEB Buffer 4 (New England Biolabs) were added. The digestion mixture was incubated at 37 °C for 2 hours. The *Cla*I enzyme was heat-inactivated by incubating at 65 °C for 20 minutes.

The digestion product was purified and desalted using Sephacryl^{TM}S-300 (Amersham). Briefly, Sephacryl^{TM}S-300 Media was mixed at a 1:1 ratio with MilliQ water. 200 μl of this mixture was added to each well of a 0.2 μm PVDF filtration plate (Corning) and spun for 2 minutes at 600 *g*. This step was repeated once. 200 μl of ddH_2O was added to each well of the filtration plate and spun for 2 minutes at 600 *g*. This step was repeated once. The digestion products were then loaded onto the Sephacryl^{TM}S-300-filled filtration plate. The purified products were collected by spinning for 2 minutes at 600 *g*.

To obtain provial/host flanking genomic fragments from as many clones as possible, genomic DNA was also digested with restriction enzyme *Eco*RI*, Xba*I*, Spe*I*, Nhe*I. The Splinkerette adapters were generated by annealing the HMSpAa with different HMSpBb oligos designed for different restriction enzymes. Because *Xba*I*, Spe*I*, Nhe*I digestion will generate the same 5’ overhang (3’-GATC-5’), these three enzymes were used to cut the genomic DNA at the same time.

2.3.3.3 First round PCR

The 5' LTR proviral flanking genomic fragments were amplified with the LTR specific primer, AB949new, and the Splinkerette primer, HMSp1. A 50 μl PCR system contains 20 μl purified ligation proucts, 1 μl AB949new (10 μM), 1 μl HMSp1 (10 μM), 5 μl 10x PCR buffer, 1.5 μl $MgCl_2$ (50 mM), 1 μl dNTPs (25 mM), 0.5 μl PlatinumTaq (5 units/μl, Invitrogen), ddH_2O 20 μl. The hot-start PCR conditions were 94 °C 1.5 minutes; 2 cycles of 94 °C 1 minute, 68 °C 30 seconds, 72 °C 1 minutes; 30 cycles of 94 °C 30 seconds, 65 °C 30 seconds, 72 °C 2 minutes; 72 °C 10 minutes.

2.3.3.4 Second round PCR

The first round of PCR product was 1:100 diluted in ddH_2O and 5 μl of the diluted product was used as the template for the second round of nested PCR. A 50 μl PCR system contains 5 μl of the diluted 1st round PCR product, 1 μl HM001 (10 μM), 1 μl HMSp2 (10 μM), 5 μl 10x PCR buffer, 1.5 μl $MgCl_2$ (50 mM), 1 μl dNTPs (25 mM), 0.5 μl PlatinumTaq (5 units/μl, Invitrogen), ddH_2O 35 μl. Hot-start PCR conditions were: 94 °C 1.5 minutes; 30 cycles of 94 °C 30 seconds, 60 °C 30 seconds, 72 °C 1.5 minutes; 72 °C 10 minutes. The nested PCR products were separated on a 1% agarose gel. The specific PCR fragments were gel purified using QIAquick Gel Extraction Kit (Qiagen) according to the manufacture's instructions.

2.3.3.5 Sequencing the splinkerette PCR products

Sequencing reactions were performed using ABI PRISM^{TM} Big DyeTerminator Cycle Sequencing Ready Reaction Kits (PE Applied Biosystems) according to the manufacturer's instructions. A 10 μl sequencing mix contains 5 μl gel purified PCR product, 1 μl of HM002 or HMSp3 primer (5 μM) and 4 μl Big Bye. The sequencing conditions were 94 °C for 1.5 minutes; 40 cycles of 94 °C 30 seconds, 55 °C 30 seconds, 60 °C 4 minutes.

After the sequencing reaction, 10 μl of MilliQ water was added to each well of the 96-well plate. 50 μl of Precipitation Mix (100 ml 96% ethanol, 2 ml

Na_2OAC (3 M, pH 5.2), 4 ml EDTA (0.1 mM, pH 8.0)) was then added to each well. The precipitated sequencing products were collected by centrifugation at 4000 rpm at 4 °C for 25 minutes. The supernatant was discarded and the precipitates were washed with 100 µl of chilled 70% ethanol followed by centrifuging at 4000 rpm at 4 °C for 10 minutes. The ethanol was discarded and the samples were dried at 65°C for 2 minutes. The sequencing reactions were run on an ABI PRISM™ 3730 DNA sequencer (Perkin Elmer).

Splinkerette PCR primers:
AB949new: 5'-GCT AGC TTG CCA AAC CTA CAG GTG G-3'
HM001: 5'- GCC AAA CCT ACA GGT GGG GTC TTT-3'
HMSp1: 5'-CGA AGA GTA ACC GTT GCT AGG AGA GAC C-3'
HMSp2: 5'-GTG GCT GAA TGA GAC TGG TGT CGA C-3'

Splinkerette sequencing primers:
HM002: 5'-ACA GGT GGG GTC TTT CA-3'
HMSp3: 5'-GGT GTC GAC ACT AGT GG-3'

2.4 RNA methods

2.4.1 5' RACE

2.4.1.1 Total RNA extraction

Total RNA was extracted from ES cells grown on gelatinized 6-well tissue culture plate using RNAqueous™ Kits (Ambion) according to the manufacturer's protocol. 5 µg of total RNA was treated with 1 µl amplification grade DNase I (1 unit/µl, Invitrogen) in a 10 µl volume for 15 minutes at room temperature to eliminate the residual genomic DNA. After the DNase I treatment, 1 µl of EDTA (25 mM) was added to each reaction, and the reaction mixture was incubated at 65 °C for 15 minutes to heat-inactivate the DNase I.

2.4.1.2 First strand cDNA synthesis

3 µl *lacZ*-GSP1primer (10 µM, dissolved in DEPC-treated water) and 9 µl DEPC-treated water was added to the reaction to make up the volume to 25

μl. The RNA template was denatured by incubation at 65°C for 10 minutes and then placed on ice for 1 minute. 1 μl dNTPs (10 mM, Invitrogen), 10 μl 5X first-strand buffer, 5 μl DTT (0.1 M), 1 μl Superscript™ II (5 units/μl), 8 μl DEPC-treated water were added to denatured RNA template. The mixture was incubated at 50 °C for 1 hour. The retro-transcriptase was heat-inactivated by incubation at 70 °C for 15 min. After that, 1 μl of Ribonuclease H (2 U/μl, Invitrogen) was added. The mixture was incubated at 37 °C for 30 minutes to destroy the RNA template. The synthesized first strand cDNA was purified using QIAquick PCR purification kit (Qiagen). If first strand cDNA was synthesized on 96-well PCR plates, the samples were purified using Sephacryl™S-300 (Amersham) as described before.

lacZ-GSP1: 5'-GGG CCT CTT CGC TAT TAC GC-3'

2.4.1.3 TdT tailing

8 μl 5X TdT buffer, 2 μl dCTP (4 mM) and 1 μl TdT enzyme (Invitrogen) were added to 30 μl purified first strand cDNA. The samples were incubated for 10 minutes at 37 °C. After the reaction, the TdT enzyme was heat-inactivated by incubating the samples for 10 min at 65 °C.

2.4.1.4 First round PCR

The 5' RCAE products were amplified with the *lacZ* specific primer, *lacZ*-GSP2, and the 5' RACE Abridged Anchor Primer (AAP, Invitrogen). A 50 μl PCR system contains 10 μl purified dC-tailed cDNA, 1 μl *lacZ*-GSP2 (10 μM), 1 μl AAP (10 μM), 5 μl 10x PCR buffer, 1.5 μl $MgCl_2$ (50 mM), 1 μl dNTPs (25 mM), 0.5 μl PlatinumTaq (5 units/μl, Invitrogen) and 30 μl ddH_2O. The hot-start PCR conditions were 94 °C 1.5 minutes; 35 cycles of 94 °C 30 seconds, 55 °C 30 seconds, 72 °C 2 minutes; 72 °C 10 minutes.

2.4.1.5 Second round PCR

First-round PCR products were 1:100 diluted using ddH_2O. 5 μl of the diluted PCR product was used as the template for the second round of nested PCR. A 50 μl PCR system contains 5 μl diluted 1st round PCR product, 1 μl *lacZ*-

GSP3 (10 μM), 1 μl Abridged Universal Amplification Primer (AUAP, Invitrogen), 5 μl 10X PCR buffer, 1.5 μl $MgCl_2$ (50 mM), 1 μl dNTP (25 mM), 0.5 μl Platinum Taq (5 units/μl, Invitrogen), 35 μl ddH_20. The hot-start PCR conditions were 94 °C 1.5 minutes; 35 cycles of 94 °C 30 seconds, 55 °C 30 seconds, 72 °C 2 minutes; 72 °C 10 minutes. 10 μl of the nested PCR products were loaded on a 1.0 % agarose gel.

2.4.1.6 Sequencing the 5' RACE product

If the nested-PCR was performed on a small scale, the nested-PCR product was purified using QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. If the nested-PCR was performed in a 96-well plate format, 10 μl of the nested-PCR product was treated with 1U each of Exonuclease I (Exo I, NEB) and Shrimp Alkaline Phosphatase (SAP, Amersham) for one hour at 37 °C to get rid of the unused primers and dNTPs. After the reaction, the mixture was incubated at 95 °C for 15 minutes to heat-inactivate the enzymes, and 5 μl was used for sequencing.

Sequencing reaction was performed using ABI PRISM™ Big DyeTerminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) according to the manufacturer's instructions. A 10 μl sequencing mix contains 5 μl purified PCR product, 1 μl of *SA*-seq primer (5 μM) and 4 μl Big Bye. The sequencing conditions were 94 °C for 1.5 minutes; 40 cycles of 94 °C 30 seconds, 55 °C 30 seconds, 60 °C 4 minutes.

After sequencing reaction, 10 μl of MilliQ water was added to each well of the 96-well plate. 50 μl of Precipitation Mix (100 ml 96% ethanol, 2 ml Na_2OAC (3 M, pH 5.2), 4 ml EDTA (0.1 mM, pH 8.0)) was then added to each well. The sequencing products were precipitated by centrifugation at 4000 rpm at 4 °C for 25 minutes. The supernatant was discarded and the precipitates were washed with 100 μl of chilled 70% ethanol followed by centrifuging at 4000 rpm at 4 °C for 10 minutes. The ethanol was discarded and the samples were dried at 65°C for 2 minutes. The sequencing reactions were run on an ABI PRISM™ 377 DNA sequencer (Perkin Elmer).

5’ RACE PCR primers:
lacZ-GSP2: 5’-ATG TGC TGC AAG GCG ATT AAG-3’
SA-GSP3: 5’-GTT GTA AAA CGA CGG GAT CCG CCA T-3’

5’ RACE sequencing primers:
SA-seq: 5’-TGTCAC AGA TCA TCA AGC TTA TC-3’

2.4.2 RT-PCR

2.4.2.1 First strand cDNA synthesis

Total RNA was prepared using an RNeasy® Mini Kit (Qiagen). The total DNA from each sample was quantified using Spectrophotometer (Beckman). 5 μg total RNA of each sample was used for each reaction, DEPC-treated water was added to each sample to bring up the final volume to 24 μl. 1 μl of Oligo-dT primer (10 μM) was added to each reaction. The RNA template was denatured by incubation at 65°C for 10 minutes and then placed on ice for 1 minute. 1 μl dNTPs (10 mM, Invitrogen), 10 μl 5X first-strand buffer, 5 μl DTT (0.1 M), 1 μl Superscript^{TM} II (5 units/μl, Invitrogen) and 8 μl DEPC-treated water were added to denatured RNA template. The retro-transcriptase was heat-inactivated by incubation at 70 °C for 15 min. After that, 1 μl of Ribonuclease H (2 U/μl, Invitrogen) was added. The mixture was incubated at 37 °C for 30 minutes to destroy the RNA template. The resultant cDNA was diluted at a ratio of 1:5 with ddH_2O and 5 μl was used for each PCR reaction.

2.4.2.2 RT-PCR

The first strand cDNA was amplified with the gene-specific primers designed. A 50 μl PCR system contains 5 μl diluted cDNA, 1 μl Forward Primer (10 μM), 1 μl Reverse Primer (10 μM), 5 μl 10x PCR buffer, 1.5 μl $MgCl_2$ (50 mM), 0.5 μl dNTPs (25 mM), 0.5 μl PlatinumTaq (5 units/μl, Invitrogen) and 35.5 μl ddH_2O. The hot-start PCR conditions were 94 °C 1.5 minutes; 25-35 cycles (depends on the primers) of 94 °C 30 seconds, 55-65 °C (depends on the primers) 30 seconds, 72 °C 1 minute; 72 °C 10 minutes.

Oligo-dT primer: GGC CAC GCG TCG ACT AGT AC $(T)_{17}$

Other germ layer and cell lineage specific marker: Table 2.4

Table 2.4: RT-PCR primers used for *in vitro* differentiation assay.

Gene Name	Forward Primer	Reverse Primer	Annealing Temperature	Length of PCR product	Reference
Alk-3	TCACCGAAAGCCCAGCTACG	TCACCGAAAGCCCAGCTACG	55°C	700 bp	Wiles MV, Johansson BM. *Exp Cell Res.* 1999 Feb 25;247(1):241-8.
Brachyury	ATGCCAAAGAAAGAAACGAC	AGAGGCTGTAGAACATGATT	55°C	836 bp	
Fyn	CAACCGGGGAAACTGGTTAC	GCTCATGTACTCCGTGACGA	55°C	645 bp	
Goosecoid	GCACCATCTTCACCGATGAG	AGGAGGATCGCTTCTGTCGT	55°C	179 bp	
Nodal	TCACCGTCCCCTCTGGCGTA	ACTCCTCCCCCACAGGGTTA	60°C	773 bp	
Noggin	TGGCGGCCGCCTTCCCAAGT	AGCCCGGGGGATCCATCAAG	60°C	365 bp	
Pax-6	CAGTCACAGCGGAGTGAATC	CGCTTCAGCTGAAGTCGCAT	55°C	658 bp	
Scleraxis	GTGGACCCTCCTCCTTCTAATTCG	GACCGCACCAACAGCGTGAA	63°C	375 bp	Kramer J, Hegert C, Guan K, Wobus AM, Muller PK, Rohwedel *J. Mech Dev.* 2000 Apr;92(2):193-205.
Pax-1	TTCTCGGTGTTTGAAGGTCATTGCCG	GATGGAAGACTGGGCGGGTGTGAA	60°C	318 bp	
Sox-9	TCTTTCTTGTGCTGCACGCGC	TGGCAGACCAGTACCCGCATCT	57°C	135 bp	
Aggrecan	TCCTCTCCGGTGGCAAAGAAGTTG	CCAAGTTCCAGGGTCACTGTTACCG	60°C	270 bp	
Collagen II	AGGGGTACCAGGTTCTCCATC	CTGCTCATCGCCGCGGTCCTA	60°C	432 bp (splice variant A) and 225 bp (splice variant B)	
b-Tubulin	GGAACATAGCCGTAAACTGC	TCACTGTGCCTGAACTTACC	54°C	317 bp	
HPRT	GCCTGTATCCAACACTTCG	AGCGTCGTGATTAGCGATG	63°C	507 bp	
PECAM	GTCATGGCCATGGTCGAGTA	CTCCTCGGCATCTTGCTGAA	55°C	260 bp	Vittet D, Prandini MH, Berthier R, Schweitzer A, Martin-Sisteron H, Uzan G, Dejana E. *Blood.* 1996 Nov 1;88(9):3424-31.
Flk-1	TCTGTGGTTCTGCGTGGAGA	GTATCATTTCCAACCACCCT	55°C	269 bp	
Tie-1	CCTTCCTACCTGCTA	CCACTACACCTTTCTTTACA	55°C	441 bp	
Tie-2	CTCACTGCCCTCCTGACTGG	CGATGTACTTGGATATAGGC	55°C	228 bp	
VE-Cadherin	GGATGCAGAGGCTCACAGAG	CTGGCGGTTCACGTTGGACT	55°C	226 bp	
HPRT	GCTGGTGAAAAGGACCTCT	CACAGGACTAGAACACCTGC	55°C	248 bp	
a-Cardiac myosin heavy chain	CTGCTGGAGAGGTTATTCCTCG	GGAAGAGTGAGCGGCGCATCAAGG	64°C	301 bp	Fassler R, Rohwedel J, Maltsev V, Bloch W, Lentini S, Guan K, Gullberg D, Hescheler J, Addicks K, Wobus. *AM.J Cell Sci.* 1996 Dec;109 (Pt 13):2989-99.
b-Cardiac myosin heavy chain	TGCAAAGGCTCCAGGTCTGAGGGC	GCCAACACCAACCTGTCCAAGTTC	64°C	205 bp	
Myosin light chain isoform 2V	TGTGGGTCACCTGAGGCTGTGGTTCAG	GAAGGCTGACTATGTCCGGGAGATGC	64°C	189 bp	
Atrial natriuretic factor	TGATAGATGAAGGCAGGAAGCCGC	AGGATTGGAGCCCAGAGTGGACTAGG	64°C	203 bp	
Cardiac-specific a1-subunit of the L-type calcium channel	GTTCCTGAAGGAGGTGTGCTGGACG	AAAGGCAGTTCCCATGCCGG	62°C	183 bp	
Skeletal muscle-specific a1-subunit of the L-type calcium channel	GATCACCAGCCAATAGAAGACC	GGCGAGGTCATGGACGTGGACG	62°C	200 bp	
b-Tubulin	GGAACATAGCCGTAAACTGC	TCACTGTGCCTGAACTTACC	64°C?	317 bp	
NFL	CCAGGAAGAGCAGACAGAGGT	GTTGGGAATAGGGCTCAATCT	59°C	302 bp	Rohwedel J, Kleppisch T, Pich U, Guan K, Jin S, Zuschratter W, Hopf C, Hoch W, Hescheler J, Witzemann V, Wobus AM. *Exp Cell Res.* 1998 Mar 15;239(2):214-25.
NFH	AGGACCGTCATCAGGCAGACATTGC	CTTCTGTCACTCCTTCCGTCACCCG	59°C	368 bp	
Synaptophysin	TACCGAGAGAACAACAAAGGGC	GCCTGTCTCCTTGAACACGAAC	60°C	287 bp	
Tau	CCGCACTCCCCCTAAGTCACCATC	TGCCGTGGAGATGTGTCCCCAGAC	60°C	440 bp(splice variant A) and 578 bp (splice variant B)	
S-laminin	TGGCTGTCTACCTGGCATCTGG	GCGACCACCATCTTGAGAACCC	58°C	187 bp	
AChR e-unit	ATTTCTGGCTTGGTGCTGCTCGC	GAGTCGTTGGCGTCCTCAAAGATACG	59°C	246 bp	

Table 2.4 (cont): RT-PCR primers used for *in vitro* differentiation assay.

Gene Name	Forward Primer	Reverse Primer	Annealing Temperature	Length of PCR product	Reference
68 kDa neurofilament protein (=NFL)	CCAGGAAGAGCAGACAGAGGT	GTTGGGAATAGGGCTCAATCT	59°C	302 bp	Rohwedel J, Guan K, Zuschratter W, Jin S, Ahnert-Hilger G, Furst D, Fassler R, Wobus AM. *Dev Biol.* 1998 Sep 15;201(2):167-84.
200 kDa neurofilament protein (=NFH)	AGGACCGTCATCAGGCAGACATTGC	CTTCTGTCACTCCTTCCGTCACCCG	59°C	368 bp	
Synaptophysin	TACCGAGAGAACAACAAAGGGC	GCCTGTCTCCTTGAACACGAAC	60°C	287 bp	
Tau	CCGCACTCCCCCTAAGTCACCATC	TGCCGTGGAGATGTGTCCCCAGAC	60°C	440 bp(splice variant A) and 578 bp (splice variant B)	
Brachyury	GAGAGAGAGCGAGCCTCCAAAC	GCTGTGACTGCCTACCAGAATG	59°C	230 bp	
Pax-6	GCTTCATCCGAGTCTTCTCCCTTAG	CCATCTTTGCTTGGGAAATCCG	59°C	312 bp	
Mash-1	CTCGTCCTCTCCGGAACTGATG	CGACAGGACGCCCGCCTGAAAG	62°C	301 bp	
BMP-4	ATTCTCTGGGATGCTGCTGAGG	CCGAGCCAACACTGTGAGGAGT	59°C	114 bp	
Wnt-1	GATTGCGAAGATGAACGCTGTTTC	TCCTCCACGAACCTGTTGACGG	54°C	266 bp	
S-laminin	TGGCTGTCTACCTGGCATCTGG	GCGACCACCATCTTGAGAACCC	58°C	187 bp	
b-Tubulin	GGAACATAGCCGTAAACTGC	TCACTGTGCCTGAACTTACC	60°C	317 bp	
HPRT	GCCTGTATCCAACACTTCG	AGCGTCGTGATTAGCGATG	59°C	507 bp	
B7-1	ATGGCTTGCAATTGTCAGTT	ATCAGGAGGGTCTTCTGGGGGT	55°C	728 bp	Ling V, Munroe RC, Murphy EA, Gray GS. *Exp Cell Res.* 1998 May 25;241(1):55-65.
B7-2	ATCGCCAACTTCAGTGAACC	TCTCACTGCCTTCACTCTGC	55°C	525 bp	
CD28	ACTCAGGCTGCTGTTCTTGG	TCGTTGTCTAGGTAAGGCGG	55°C	375 bp	
CTLA-4	CACAACACTGATGAGGTCCG	TGAGTTCCACCTTGCAGAGG	55°C	210 bp	
b-Actin	GTCGTCGACAACGGCTCCGGCATGTG	CATTGTAGAAGGTGTGGTGCCAGAT	55°C	252 bp	
K18	TTGTCACCACCAAGTCTGCC	TTTGTGCCAGCTCTGACTCC	60°C	213 bp	Bagutti C, Wobus AM, Fassler R, Watt FM. *Dev Biol.* 1996 Oct 10;179(1):184-96.
K14	GTGTCCACTGGCGATGTGAACGTGG	GCTGCCGCAGTAGCGACTCTACTGT	60°C	330 bp	
K10	CGCAAGGATGCTGAAGAGTGGTTC	TGGTACTCGGCGTTCTGGCACTCGG	60°C	278 bp	
Involucrin	GGTGTACAGAAGCTTCCAAGATGTCC	GGCATTGTGTAGGATGTGGAGTTGG	60°C	150 bp	
actin	GTTTGAGACCTTCAACACCCC	GTGGCCATCTCCTGCTCGAAGTC	60°C	320 bp	
Cx40	CCACGGAGAAGAATGTCTTCA	TGCTGCTGGCCTTACTAAGG	N.D.	447 bp	Oyamada Y, Komatsu K, Kimura H, Mori M, Oyamada M. *Exp Cell Res.* 1996 Dec 15;229(2):318-26.
Cx43	TGGGGGAAAGGCGTGAG	CTGCTGGCTCTGCTGGAAGGT	N.D.	1.3 kb	
Cx45	ATCATCCTGGTTGCAACTCC	CTCTTCATGGTCCTCTTCCG	N.D.	168 bp	
MHC-a	CTGCTGGAGAGGTTATTCCTCG	GGAAGAGTGAGCGGCGCATCAAGG	N.D.	302 bp	
MHC-b	TGCAAAGGCTCCAGGTCTGAGGGC	GCCAACACCAACCTGTCCAAGTTC	N.D.	205 bp	
MLC-2V	GCCAAGAAGCGGATAGAAGG	CTGTGGTTCAGGGCTCAGTC	N.D.	499 bp	
TTR	CCTCTGATGGTCAAAGTCCTGGATGCTG	CCTGGTCCTCCTGGGCTGAGTCTCTC	60°C	379 bp	Wei Wang, unpublished data
AFP	GGACATTTGTGTATAAGGAATGAAGCAAGCCC	GCAGTTACAGTTAAGCCAAAAGGCTCACACC	60°C	463 bp	
AFM	GGCAGCCCCTCAGCTCCCCAT	GGACTGAAACAGGACTCAGGCTCCTGC	60°C	465 bp	
HNF1	GGCCTCCACTCAGGCACAGAGCG	CGGAACTCTGATACAACACCAGGCTGC	60°C	488 bp	
vHNF1	CGGGAGGAGGACTGCCTCCCG	CAGGGCCCTCTCCTGGGCTCCC	60°C	574 bp	
HNF4	CGGCTGCGGTCACAGGTGCAAG	AGGTGCTCTTCTGAGGGTATGAGCCAGC	60°C	554 bp	
HNF3b	GGCCAGACCACGCGAGTCCTACG	TGAGCCGCTCATGCCCGCCAT	60°C	377 bp	
Oct3/4	GTGTAAGCTGCGGCCCCTGCTGG	GCTTCCATAGCCTGGGGTGCCAAAGT	60°C	388 bp	
EKLF	CTGGGACCTGGGACTGTGGCCAC	GGCCCATCTTTTGGGATACGGTCC	60°C	449 bp	
FGF5	CTGCAGAGTGGGCATCGGTTTCC	GCTCGGACTGCTTGAACCTGGGTAGG	60°C	396 bp	
GATA4	GGACACTACCTGTGCAATGCCTGTGG	GACAGGAGATGCATAGCCTTGTGGGG	60°C	532 bp	
Msx3	CCACACAGAGCACGGACCACTCCCCT	CCTGAGCCTAACCAAGAGGTTAGGGCTT	60°C	598 bp	
Nkx2.5	GCCTGGCGTCTGGGGACCTGTCTG	TGCGCGTGGTCTCTCGGCGC	60°C	327 bp	
PECAM	CCAGTGCAGAGCGGATAATTGCCATTCC	TAAGGTGGCGATGACCACTCCAATGAC	60°C	428 bp	