CHAPTER 2:

MATERIALS AND METHODS

2.1 Vectors

2.1.1 Gene targeting vectors

The following vectors were obtained from Pentao Liu and used in the construction of targeting and retrieval constructs.

PL611 (Retrieval vector) (Appendix A.1):

PL611 is a retrieval vector derived from pBR322 (Covarrubias et al., 1981) which can support replication of relatively large pieces of mammalian genomic DNA in *E. coli*. This plasmid serves as the PCR template for generating the retrieval vector.

Bsd cassette (*I-Sce*I-*EM7-Bsd-I-Ceu*I selection plasmid) (Appendix A.2):

The *Bsd* (blasticidin) cassette serves as the PCR template for generating the 5' targeting vector. It contains two rare enzyme restriction sites (*I-Sce*I and *I-Ceu*I) flanking the Bsd resistance coding sequence (Bsd^R).

Neo cassette (*lox*P-*F3-PGK-EM7-Neo-F3* selection plasmid) (Appendix A.3):

The *Neo* (neomycin) cassette serves as the PCR template for generating the 3' targeting vector. It contains a single *lox*P site and two *F3* sites flanking the Neo resistance coding sequence (Neo^R).

PL613 (*I-Sce*I-*FRT-SA-IRES-lacZ-PGK-EM7-Puro-FRT-lox*P-*I-Ceu*I plasmid) (Appendix A.4):

PL613 is the *lacZ* reporter cassette plasmid which contains the bacterial *lacZ* gene with a nuclear localisation signal and the puromycin resistance coding sequence ($Puro^R$). The *lacZ* and *Puro* selection marker are flanked by two *FRT* sites. Restriction digestion of this plasmid with two rare enzyme restriction sites (*I-Sce*I and *I-Ceu*I) would release the *lacZ* reporter module.

Cm-TK cassette (*MC1-TK-Cm* plasmid) (Appendix A.5):

The *Cm-TK* cassette contains the chloramphenicol resistance coding sequence (Cm^R) and the negative selection marker, *MC1-TK*. *Cm-TK* cassette is flanked by two 600 bp homology regions to PL611, therefore *MC1-TK* can be added to the targeting vector backbone by recombineering.

pSim6/pSim18 (Appendix A.6):

pSim6 was constructed by recombination of the λ-defective prophage into pSC101 plasmid backbone (Datta et al., 2006). The ampicillin resistance coding sequence (Amp^R) is present as a substitute for the *rexAB* genes. pSim18 was constructed by replacing the Amp^R in pSim6 with either the Bsd^R or hygromycin-resistant coding sequences (Hyg^R) using the recombineering functions expressed from the plasmid.

2.1.2 Over-expression vectors

PL417 (*MSCV-IRES-eGFP* plasmid) and PL419 (*MSCV-Bcl11a* cDNA-*IRES-eGFP* plasmid) were obtained from Pentao Liu. *MSCV-Bcl11b* cDNA-*IRES-eGFP* plasmid was constructed as follows: Firstly, two pairs of primers were designed for amplification of *Bcl11b* cDNA (Appendix A.7A) from total mouse brain oligo-dT cDNA library (constructed as detailed in Chapter 2.6.3). PCR amplification was carried out using Pwo master (Roche) and performed using PTC-225 PCR machine (Peltier Thermal Cycler) with the following settings: 94°C for 2 min, this was followed by 35 cycles of 94°C for 30 sec, 52-62°C for 30 sec and 72°C for 45 sec (*Bcl11b* exons 1-2-3) or 2.5 min (*Bcl11b* exons 3-4). This was then followed by 72°C for 5 min. PCR products, containing *Bcl11b* exons 1-2-3 (529 bp; Appendix A.7B) and exons 3-4 (2240 bp; Appendix A.7C) respectively, were run through a 1% agarose gel and appropriate bands were purified using Qiagen gel extraction kit. 4 µl of purified PCR products were used for cloning into pCR-BluntII-TOPO vector and transformed into TOP10 cells using manufacturer's protocol (Invitrogen). 1.5 µg of each plasmid were digested with *Bgl*II and *Cla*I (*Bcl11b* exons 1-2-3; Appendix A.7D) or *Cla*I and *Eco*RI (*Bcl11b* exons 3-4; Appendix A.7E) and ligated with 1.5 µg of PL417 that has been cut with *Bgl*II and *Eco*RI. 2 µl of ligation products were transformed into DH10B cells and transformants were selected on LB-

Amp plates. Plasmids obtained from minipreparations of Amp^R colonies were checked by restriction digestion (Appendix A.7F) and sequencing to ensure that there were no mutations in the *Bcl11b* cDNA.

2.1.3 *Bcl11-lacZ* reporter conditional null vectors

2.1.3.1 Construction of targeting and retrieval vectors

A novel PCR method using 100 mers PAGE-purified primers to construct the targeting and retrieval vectors was employed. These primers consist of 80 bases of homologous sequence to designated *Bcl11* targeted/retrieval regions (Ensembl *Mus musculus,* Sanger Institute) and 20 bases of complementary sequence to targeting/retrieval vector plasmids (Appendix A.1-A.3). Primers used are listed in Table 2.1. The templates for PCR amplification were prepared as follows: The linear retrieval vector (PL611, *Eco*RI and *Bam*HI digested; Appendix A.1), *loxP-F3-Neo-F3* (*Neo* cassette, *Not*I and *Sal*I digested; Appendix A.2) and *I-Sce*I-*Bsd-I-Ceu*I (*Bsd* cassette, *Eco*RI and *Bam*HI digested; Appendix A.3) selection cassettes were run through a 1% agarose gel and bands corresponding to 560 bp (*Bsd),* 2 kb (*Neo*) and 3 kb (PL611) were excised and purified using Qiagen purification kit. To avoid the background from uncut cassettes of the PCR templates, 1 ng of the restriction digested and gel-purified retrieval vector and the two selection cassettes were transformed into DH10B cells. If there were any background drug resistant colonies, the cassettes were re-purified and tested again until they were clean.

One nanogram of each of the above linear purified products was used as a template for PCR reaction to generate PCR products for targeting or retrieving. PCR amplification was carried out using Extensor Hi-Fidelity PCR Master Mix 2 (ABgene). 25 µl of the master mix was added to 1 µl of template (1 ng), 2 µl of each primer (10 µM) and 20 µl of PCR-grade water. PCR was performed using PTC-225 PCR machine (Peltier Thermal Cycler) with the following settings: 94^{o}C for 4 min, followed by 35 cycles of 94^{o}C for 30 sec, 60^{o}C for 30 sec and 68^{o}C for 1 min (*Bsd*) or 2-3 min (*Neo* and retrieval backbone). This was then followed by 68^{o}C for 5 min. After PCR reactions, 0.5 µl of exonuclease I (10U, New England Biolabs, NEB) was added per 50 µl of PCR products and incubated at 37^{o}C for 1 hour followed by heat inactivation at 80^{o}C for 20 min. The

PCR products were then purified using Qiagen mini-preparation columns and eluted in 50 µl of PCR-grade water to obtain the targeting and retrieval vectors.

Table 2.1. List of targeting primers used.

Targeting Primers	Primer Sequence (5'– 3')
Bcl11a-lacZ-Ret-1	GAGACTTGGTTCAAGAAACAAATATGTGTCCCTTTTGTTGTTTGTGCTAAA TTGGGAGTGAGGTTTAAAAAAAAAAATCAGATACGACTCACTATAGGGAG
Bcl11a-lacZ-Ret-2	TAATGCCTTTTATCCAAAGCCAGGAGACTTTTATCTTTTTAAGCATCGGCA AAGTAAGGTGTTTGGCTCTTACTTTTATTTTAGTGAGGGTTAATTATCG
Bcl11a-lacZ-5'-F	TCTAGCCTCACATAGGGGAGAAAGTGTATTTCTCAGTTATACTTTAAGCCC TGGCATTTTTTTAAAGTGTCTGGGACATTCTAGGGATAACAGGGTAATG
Bcl11a-lacZ-5'-R	TAACCAACAGTTTACCAGCCAACTGCAACATTTAAGGATGTAGAAGAGAC AATGGCCTAGGGACAAGGATGAGTCTAGCTTCGCTACCTTAGGACCGTTA
Bcl11a-lacZ-3'-F	AAAGTTTTATTTTATTTTATTTTTAAATGGTTATCAAATTGAATGTGAAATG TGCAAAGGCCCTGGAATGTGATGAAATATGTAAAACGACGGCCAGTGA
Bcl11a-lacZ-3'-R	TCTACTTCTTTTGGCGCCAGAATTTCATTAAATGCATCATTTTAAACAAGTA TTGTCACAAGATGAACTTCTTGCTAATGAGGAAACAGCTATGACCATG
Bcl11b-lacZ-Ret-1	CCTGCAGTTCAATCTAGTCTGGGGCGTTTCCTTAGCTTTTGCAAGCAGACA CTTCTGTTCCCTTCAGACTTGACACTGCAATACGACTCACTATAGGGAG
Bcl11b-lacZ-Ret-2	CGTGATTTTGGTGCTTTCTGTGTCTGCCCTGCATAGCCAAGGCACATTGATC TCTGCGGTTTGCTTTTGTACTGGCAAGGTTAGTGAGGGTTAATTATCG
Bcl11b-lacZ-5'-F	GCATTCCTGGGGACTGGGCTTGAGTCCTCAGACACGCTAAGCAAGCATTC CACGACGGAGCATCATAGCCATTTTTGTTACTAGGGATAACAGGGTAATG
Bcl11b-lacZ-5'-R	TGAAAATCACTCCACTGCATCCTCCCCTTGGCAACTACTGACTCTGTTGCTT TGCAAACTTAAAAAAAAAGAAAAAAAATTCGCTACCTTAGGACCGTTA
Bcl11b-lacZ-3'-F	CAGAGGCGTCTCTGTGTGGAATTCAACTTTCATGCTATGGAAGGAAAATGT TGAGGGGGGCGTGGCAAACGAGCCTCTCCTGTAAAACGACGGCCAGTGA
Bcl11b-lacZ-3'-R	CTCCTGGTAACACACAATTGCAGGATGTGGGGGCTGTTCCTAGAGTCACCC TGGAAAAGATTCTCGGGGTCCCAAGTGGGAGGAAACAGCTATGACCATG
Bcl11a_Bsd_PCR F	TCTAGCCTCACATAGGGGAGAAAGT
Bcl11a_Bsd_PCR R	CAGTTCAGCTAGTCAACTCTTTGGC
Bcl11a_Neo_PCR F	GGCTGTTTCGCCTAAAGTTTTATTT
Bcl11a_Neo_PCR R	CACAGACATAGATACTGCTGCTCTT
Bcl11b_Bsd_PCR F	GCATTCCTGGGGACTGGGCTTGAGT
Bcl11b_Bsd_PCR R	CGTCCCCTGTAGGTGTTCTTATTTT
Bcl11b_Neo_PCR F	CCTTTGCCCTACAGTGCCCATCAGC
Bcl11b_Neo_PCR R	CCTTTGCCCTACAGTGCCCATCAGC

Underlined sequences - complementary to retrieval/targeting plasmid templates

2.1.3.2 Construction of final targeting vectors

The final step in the construction is the replacement of the *Bsd* cassette on the retrieved plasmids with the *lacZ* reporter. Restriction digestion of the retrieved plasmids (1.5 µg) and PL613 (1.5 µg; Appendix A.4) with *I-Sce*I and *I-Ceu*I (NEB) was first carried out. The restriction digestion reaction of PL613 was run through a 1% agarose gel and the *lacZ* reporter cassette (7 kb band) was purified using QIAquick Gel Extraction kit (Qiagen). Next, ligation reaction of purified digestion products using 12 µl of *lacZ* reporter cassette (600 ng), 10 µl of purified retrieved plasmid (50 ng), 2.5 µl of T4 DNA

ligase buffer with 1 µl of T4 DNA ligase (NEB) was set up and incubated at room temperature for 2 hours. 5 µl of the ligation products was added to chemical competent TOP10 cells (Invitrogen) and incubated on ice for 30 min. The cells were then heat shocked at 42^{o}C for 30 sec and 250 µl of SOC was added. The transformation mixture was incubated at 37^{o}C for 1 hour before plating out the cells on a LB-Puro-Kan plate. The plate was incubated at 37^{o}C overnight and colonies are typically observed after 16-24 hours.

2.2 Recombineering

2.2.1 Using λ-phage

The replication-defective λ prophage (strain LE392) λ CI_{857} *ind1* $Cro_{TYR26amber}$ $P_{GLN59amber}$ *rex<>tetRA*, was created by combining several different mutations (Chan et al., 2007). The prophage backbone contains the three Red genes, *exo*, *bet* and *gam* and the tetracycline resistance coding sequence (Tet^R). Cells which have been successfully infected with the prophage are Tet^R and recombineering competent. BAC clones containing *Bcl11a* (bMQ-202K13 and bMQ-44A03) and *Bcl11b* (bMQ-188F4) regions were identified and obtained from Wellcome Trust Sanger Institute 129/AB2.2 BAC library. Each BAC clone was inoculated into 1 ml of LB with Cm (in a 15 ml polypropylene tube) for overnight growth at 37^{o}C with shaking at 244 G. Cells were then spun at 16,000 G for 25 sec and washed with 1 ml of SM buffer (100 mM $CaCl_2$; 8 mM $MgSO_4.7H_2O$ and 50 mM Tris-Cl, pH 7.5) before being re-suspended in 100 µl of SM buffer. 2 µl of the prophage lysate was added and the suspension was incubated at 32^{o}C for 20 min. 500 µl of LB was then added and the suspension was incubated at 32^{o}C for another 20 min. The cells were then plated out onto LB-Tet plates and incubated overnight at 32^{o}C.

Tet^R BAC clones were inoculated into 1 ml of LB with Tet and grown overnight at 32^{o}C with shaking at 243 G. The next day, 30 µl of the overnight culture was inoculated into 1 ml of LB and incubated at 32^{o}C with shaking at 243 G till OD_{600} = 0.2 (~2 hours). The cells were then heat shocked in a 42^{o}C water bath for 15 min and cooled immediately on ice for 2 min. Cells were spun down at 16,000 G for 25 sec and washed sequentially with 1 ml of ice-cold 0.1 M $CaCl_2$ and $MgCl_2$ and 1 ml of ice-cold 0.1 M

$CaCl_2$. Finally, the cell pellet was re-suspended in 50 µl of targeting/retrieval long primer PCR products (~4 µg) and 5 µl of 1 M $CaCl_2$. This mixture was heat shocked at 42°C for 2 min and cooled immediately on ice. 1 ml of LB was added and cells were incubated at 32°C with shaking at 243 G for 2 hours before plating them out on agar plates containing the appropriate antibiotics. Plates were incubated overnight at 32°C.

2.2.2 Using pSim plasmids

pSim18 (Appendix A.6) has the pSC101 replication origin which is low copy and temperature sensitive. This plasmid contains the three Red genes, *exo*, *bet* and *gam*, together with *CI857* repressor and *pL* promoter. Additionally, pSim18 has the Hyg^R cassette inserted between *CI857* and *pL* promoter. Consequently, transformants of pSim18 are selected with Hyg and these cells are now recombineering-competent. The same BAC clones as described in Chapter 2.2.1 were inoculated in 1 ml of LB with Cm (in a 15 ml polypropylene tube) for overnight growth at 37°C with shaking at 243 G. Cells were then spun at 16,000 G for 25 sec and washed thrice with ice-cold water. Cells were re-suspended in 50 µl of ice-cold water with 1 ng of pSim18 and electroporation was carried out using the following conditions: 1.75 kV, 25 F with the pulse controller set at 200 Ω. After electroporation, 1 ml of LB was added to the cuvette which was subsequently incubated at 32°C for one hour. Cells were then plated on a LB-Hyg plate and incubated overnight at 32°C.

Hyg^R BAC clones were inoculated into 1 ml of LB with Hyg and grown overnight at 32°C with shaking at 243 G. The next day, 30 µl of the overnight culture was inoculated into 1 ml of LB and incubated at 32°C with shaking at 243 G till $OD_{600} = 0.2$ (~2 hours). The cells were then heat shocked in a 42°C water bath for 15 min and cooled immediately on ice for 2 min. Following which, cells were spun at 16,000 G for 25 sec and washed thrice with ice-cold water and finally re-suspended in 50 µl of ice-cold water with 100 ng of targeting/retrieval long primer PCR products and electroporation was performed. After electroporation, 1 ml of LB was added to the cuvette which was subsequently incubated at 32°C for one hour. Cells were then plated onto agar plates containing the appropriate antibiotics and incubated overnight at 32°C.

2.2.3 Expression of Cre/Flpe recombinase

EL250 and EL350 are two DH10B derived *E. coli* strains that contain a defective prophage with *pL* operon encoding *gam* and the red recombination genes, *exo* and *bet*, under tight control of the temperature sensitive λ repressor (*CI857*). EL250 and EL350 have the Flpe recombinase or the Cre recombinase under the tight control of *AraC* and P_{BAD} respectively. Upon addition of arabinose, expression of Flpe or Cre recombinase will be induced. One colony of EL250 or EL350 was inoculated into 1 ml of LB and incubated overnight at 32°C with shaking at 243 G. The next day, 400 µl of overnight culture was inoculated into 10 ml of fresh LB and incubated at 32°C with shaking at 243 G for 2 hours. 100 µl of 10% L(+) arabinose (Sigma) was added to the culture (final concentration, 0.1%) and incubated for 1 hour at 32°C with shaking at 243 G. Following which, cells were spun at 16,000 G for 25 sec and washed thrice with ice-cold water and finally re-suspended in 50 µl of ice-cold water with 100 ng of the final targeting constructs and electroporation was performed. After electroporation, 1 ml of LB was added to the cuvette which was subsequently incubated at 32°C for one hour. Cells were then plated onto agar plates containing the appropriate antibiotics and incubated overnight at 32°C.

2.2.4 Antibiotics

Antibodies were used at the following concentration: Ampicillin (50 µg/ml); Kanamycin (30 µg/ml); Tetracycline (12.5 µg/ml); Blasticidin (50 µg/ml); Chloramphenicol (25 µg/ml) and Hygromycin (50 µg/ml).

2.3 ES cell culture

2.3.1 Culture condition

ES cell culture was performed as described before (Ramirez-Solis et al., 1993). Briefly AB2.2 (129 S7/SvEv$^{Brd\text{-}Hprtb\text{-}m2}$) wild-type ES cells and their derivatives were always maintained on SNL76/7 feeder cell layers that had been mitotically inactivated by mitomycin C treatment. ES cells were grown in M15 media (Table 2.2) and maintained at 37°C/5% CO_2. ES cell media was changed daily unless otherwise specified.

ES cells were passaged once they reached 80-85% confluence. ES cell media was changed 2 hours prior to passaging the ES cells. ES cell media was then aspirated off and the cells were washed once with 10 ml PBS. 2 ml of trypsin was added to each 9 cm plate and the plate was incubated at 37°C for 15 min. 8 ml of fresh M15 media was added to each plate to inactivate the trypsin and the cells were dispersed by repeated pipetting. The ES cell suspension was then centrifuged at 1,585 G for 5 min and the supernatant was aspirated. The cell pellet was re-suspended in 1 ml of M15 media before redistributing the ES cell suspension to four 9 cm feeder plates. The plates were maintained at 37°C/5% CO_2.

Table 2.2. Composition of 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 U/ml penicillin, 40 µg/ml streptomycin and 100 µM β-mercaptoethanol	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, 40 µg/ml streptomycin and 100 µM β-mercaptoethanol	Culture of feeder cells

2.3.2 Chemicals used for selection of ES cells

G418: Geneticin (Invitrogen) was obtained as a sterile stock solution containing 50 mg/ml of active ingredients.

Blasticidin: Blasticidin S HCl (Invitrogen), 1000X stock (5 mg/ml) was made in PBS. The stock solution was filter sterilized using a 0.2 µm syringe filter.

Puromycin: $C_{22}H_{29}N_7O_5.2HCl$ (Sigma), 100X stock (3 mg/ml) was made in MiliQ water. The stock solution was filter sterilized using a 0.2 µm syringe filter.

FIAU: 1-(2'-deoxy-2'-fluoro-b-d-arabinofuranosyl)-5-iodouracil (Oclassen Pharmaceuticals, Inc.), 1000X stock was made in PBS and NaOH was added dropwise

till the powder dissolved to give a final stock concentration of 200 mM. The stock solution was filter sterilized using a 0.2 μm syringe filter.

Trypsin: To make 5 L of trypsin, 35 g of NaCl, 5 g of D-glucose, 0.9 g of $Na_2HPO_4.7H_2O$, 1.85 g of KCl, 1.2 g of H_2PO_4, 2 g of EDTA, 12.5 g of Trypsin (1:250) and 15 g of Tris base were mixed. The pH was adjusted from 8.71 to 7.6 with HCl and phenol was added to obtain a pink colouration. Trypsin was then filter sterilized and aliquoted into 50 ml falcon tubes and stored at -20 °C.

2.3.3 Transfection of DNA into ES cells by electroporation

Prior to transfection of DNA into ES cells, ~20 μg of each targeting construct was linearized with the appropriate enzymes in a 100 μl total volume at 37°C for 2 hours. The DNA was then purified by ethanol precipitation and air-dried at room temperature. The air-dried DNA was re-suspended in sterile 1X TE (pH 8) to a final concentration of 1 μg/μl. 20 μg of DNA was used for each electroporation. ES cell electroporation was performed according to standard protocols (Ramirez-Solis et al., 1993). Briefly, ES cells (80-85% confluent) were fed 2-3 hours before harvesting. Immediately before electroporation, ES cell media was then aspirated off and the cells were washed once with 10 ml PBS. 2 ml of trypsin was added to each 9 cm plate and the plate was incubated at 37°C for 15 min. 8 ml of fresh M15 media was added to each plate to inactivate the trypsin and the cells were dispersed by repeated pipetting. The ES cell suspension was then centrifuged at 1,585 G for 5 min and the supernatant was aspirated. The cell pellet was re-suspended in PBS to a final concentration of 1 x 10^7 cells/ml. 1 x 10^7 cells were transferred into a 0.4 cm gap cuvette (Biorad) together with 20 μg of DNA. Electroporation was carried out using the Biorad gene pulser at 230 V, 500 μF. After electroporation, the cells were plated onto a 9 cm feeder plate and cultured for 10 days to allow for formation of single ES cell colonies. Drug selection was applied to the culture only 24-36 hours after electroporation.

2.3.4 Picking ES cell colonies

ES cell colonies were picked from the 9 cm feeder plates into a 96-well round bottom plate. 50 µl of trypsin was added to each well of the 96-well round bottom plate. Next, media was aspirated off the 9 cm plate and washed once with 10 ml of PBS. 8 ml of PBS was added to cover the plate and ES cell colonies were picked using a P20 Pipetman set at 10 µl and transferred into the wells of the 96-well round bottom plate (with trypsin). After completing a 96-well plate, the plate was incubated at 37°C for 15 min before adding 150 µl of fresh M15 media to each well. Next, the colonies were dispersed by vigorous pipetting using a multi-channel pipette. The ES cell suspension was then transferred to a fresh 96-well feeder plate and incubated at 37°C with 5% CO_2.

2.3.5 Passaging ES cells

When ES cells in most wells of the 96-well feeder plate reached 80-85% confluence, the plate was judged to be ready for passaging. Fresh media was added to each well 2-3 hours prior to passaging the cells. The media was then aspirated off, and the wells were washed once with PBS. Next, 50 µl of trypsin was added to each well of the 96-well plate and the plate was incubated at 37°C for 15 min. 150 µl of fresh M15 media was added to each well and the cells were dispersed by vigorous pipetting using a multi-channel pipette. The ES cell suspension was then evenly distributed to three 96-well gelatinised/feeder plates before incubating the plates at 37°C/5% CO_2.

2.3.6 Freezing ES cells

When the ES cells reached 80-85% confluence, they were ready for freezing. The media was changed 2 hours before adding trypsin to the plate. The media was then aspirated off and the plate was washed once with PBS. 50 µl of trypsin was added to each well of the 96-well plate and the plate was incubated at 37°C for 15 min. Next, 50 µl of 2X Freezing Media (60% DMEM, 20% DMSO, 20% FCS) was added to each well and the cells were dispersed by repeated pipetting using a multi-channel pipette. The plate was then sealed with tape and put into a polystyrene box with lid and frozen at -80°C.

2.3.7 Thawing ES cells

To thaw frozen ES cell clones, the 96-well plate was taken out of the -80^{o}C freezer and placed immediately into the 37^{o}C incubator. After ensuring that all the wells had thawed completely, the required clones were transferred to individual wells of a 24-well feeder plate containing 2 ml of M15 media. For maximum recovery of cells, another 200 µl of M15 media was added to rinse each well of the 96-well plate and the cell suspension was transferred into corresponding wells of the 24-well feeder plate. The plates were then incubated at 37^{o}C/5% CO_2.

2.3.8 Generation of targeted ES cell lines

Bcl11a-lacZ (AB2.2 targeted with *Bcl11a-lacZ* construct):

Twenty micrograms of *Bcl11a-lacZ* construct was linearized with *I-Ppo*I and electroporated into AB2.2 cells. 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 for long range PCR analysis. The expected sizes of the PCR products were 6.435 kb for 5' PCR and 3.446 kb for 3' PCR. The correctly targeted clones were expanded.

Bcl11b-lacZ (AB2.2 targeted with *Bcl11b-lacZ* construct):

Twenty micrograms of *Bcl11b-lacZ* construct was linearized with *I-Ppo*I and electroporated into AB2.2 cells. 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 for long range PCR analysis. The expected sizes of the PCR products were 7.141 kb for 5' PCR and 3.969 kb for 3' PCR. The correctly targeted clones were expanded.

2.4 Mouse techniques

2.4.1 Animal husbandry

Mice were treated in accordance with local ethical committee and the UK Animals (Scientific Procedures) Act, 1986 and all procedures were approved by the British Home Office Inspectorate. *Bcl11-lacZ* reporter lines were generated as detailed

above (Chapter 2.2). *Bcl11* conditional knockout mice were generated by Pentao Liu. Briefly, exon 1 and exon 4 of *Bcl11a* and *Bcl11b* respectively were flanked by *lox*P sites; upon expression of Cre recombinase, the intervening sequences between the *lox*P sites would be deleted, resulting in a null allele. The Rosa26-CreERT2 line, where Cre expression is driven by *Rosa26* promoter, was a kind gift from David Adams. In the Rosa26-CreERT2 line, Cre has been fused to the ligand-binding domain of a mutated human estrogen receptor (ERT) that recognizes tamoxifen or its derivative 4-hydroxytamoxifen. Therefore, Cre-estrogen receptor (CreERT) fusion proteins are retained in the cytoplasm but translocated to the nucleus on addition of the synthetic ligand. The BLG-Cre line, where the Cre transgene is driven by the promoter of the milk gene *β-lactoglobulin* (BLG), was obtained from Christine Watson (Selbert et al., 1998). Virgin female mice, 8 to 14 weeks old were mated and plug checked to confirm mating and pregnancy was confirmed post-mortem to avoid pseudo-pregnancies. At least three mice of each genotype and each time point were analysed.

2.4.2 Tamoxifen preparation and injection

One gram of tamoxifen-free base (Sigma) was re-suspended in 5 ml of ethanol and then dissolved in 50 ml of sunflower oil to obtain a final stock concentration of 20 mg/ml. This stock solution was then sonicated on ice for 2 min (at 15 sec interval) to ensure that the tamoxifen had completely dissolved. 1 mg of tamoxifen-free base in sunflower oil was administrated to each mouse by intraperitoneal injection for 3 consecutive days. Food mash was given to injected mice at the end of third injection and their weights were closely monitored.

2.5 DNA methods

2.5.1 Extraction of DNA from BAC clones

BAC clones were inoculated in 5 ml of LB with Cm at 37^{o}C with shaking at 244 G. The overnight culture was spun down at 16,000 G for 5 min and the pellet was washed with 1 ml of STE (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8). The pellet was then re-suspended in 250 µl of P1 buffer (Qiagen) and clones were lysed with an equal volume of P2 buffer (Qiagen). Next, 350 µl of N3 (Qiagen) was added and the cell lysates were

spun at 16,000 G for 5 min. The supernatant was transferred to a fresh eppendoff tube and DNA was precipitated with 0.7 volumes of isopropanol. The DNA was pelleted by spinning at 16,000 G for 10 min and washed once with 1 ml of 70% ethanol. Finally, the DNA was air dried before re-suspending in an appropriate volume of TE buffer.

2.5.2 Extraction of DNA from ES cells

ES cells were incubated in 50 µl of lysis buffer [10 mM Tris (pH 7.5), 10 mM EDTA (pH 8), 10 mM NaCl, 0.5% Sarcosyl, and 1 mg/mL Proteinase K (added fresh)] at 65^{o}C overnight in a humidified chamber. Next, 100 µl of freshly prepared solution of 75 mM NaCl in ethanol was added and the suspension was incubated at room temperature for 30 min. The cell suspension was then spun at 3,000 G for 5 min and washed twice with 70% ethanol. The DNA was air dried before re-suspended in an appropriate volume of TE buffer.

2.5.3 Extraction of DNA from tissues

Tissues (ear or tail biopsies) were incubated in 600 µl of lysis buffer [50 mM Tris (pH 8), 100 mM NaCl, 25 mM EDTA (pH 8), 0.5% SDS, and 0.5 mg/ml Proteinase K (added fresh)] at 65^{o}C for 2 hours. 500 µl of buffered phenol/chloroform/isoamyl alcohol (25:24:1 parts by volume) was then added and the suspension was mixed well before centrifuging at 16,000 G for 5 min. The aqueous phase was then carefully removed and added to 420 µl of isopropanol to precipitate the DNA. The DNA pellet was washed once with 1 ml of 70% ethanol and air dried before being re-suspended in an appropriate volume of TE buffer.

2.5.4 Polymerase chain reaction (PCR)

2.5.4.1 Long range PCR

PCR amplification was carried out using Extensor Hi-Fidelity PCR Master Mix 2 (2X, ABgene). 25 µl of the master mix was added to 1 µl of template (1-5 ng), 2 µl of each primer (10 µM) and 20 µl of PCR-grade water. PCR was performed using PTC-225 PCR machine (Peltier Thermal Cycler) with the following settings: 94^{o}C for 4 min, this was followed by 35 cycles of 94^{o}C for 30 sec, 60^{o}C for 30 sec and 68^{o}C for 5 min (5’

PCR) or 3 min (3' PCR). This was then followed by 68^{o}C for 10 min. The PCR reaction was mixed with 6X loading dye and visualised on a 0.8% agarose gel. Primers used are shown in Table 2.3.

2.5.4.2 Genotyping PCR

PCR amplification was carried out using Extensor Hi-Fidelity PCR Master Mix 2 (2X, ABgene). 5 µl of the master mix was added to 1 µl of template (1-5 ng), 1 µl of each primer (10 µM) and 2 µl of PCR-grade water. PCR was performed using PTC-225 PCR machine (Peltier Thermal Cycler) with the following settings: 94^{o}C for 4 min, this was followed by 35 cycles of 94^{o}C for 30 sec, 60^{o}C for 30 sec and 68^{o}C for 45 sec. This was then followed by 68^{o}C for 10 min. The PCR reaction was mixed with 6X loading dye and visualised on a 1% agarose gel. Primers used are shown in Table 2.3.

Table 2.3. List of genotyping primers used.

Long range PCR primers	Primer sequence (5' – 3')	Size (bp)
Bcl11a-5'-Fwd	GAGACAAGAACAGGTGCAAGAGTGGATT	6,435
Bcl11a-5'-Rev	CAAGGAAACCCTGGACTACTGCGCCCTA	
Bcl11a-3'-Fwd	GAAAGAACCAGCTGGGGCTCGACTAGAG	3,446
Bcl11a-3'-Rev	CAGCGAGGTCCCCTTTCTCACTAAAAAT	
Bcl11b-5'-Fwd	GCTTAACTGGACACCATGTGCATACCCC	7,141
Bcl11b-5'-Rev	CAAGGAAACCCTGGACTACTGCGCCCTA	
Bcl11b-3'-Fwd	GAAAGAACCAGCTGGGGCTCGACTAGAG	3,969
Bcl11b-3'-Rev	CTGTAAGAGACAAACCCTCTAGGCAAAC	
Genotyping PCR primers	**Primer sequence (5' – 3')**	**Size (bp)**
Bcl11a-lacZ-Fwd	GCTTGCTTTGGAATATGAATGTTTG	363 (Wt); 437 (Mut)
Bcl11a-lacZ-Rev1	CATATATGGGGTTTATGGAGTAACC	
Bcl11a-lacZ-Rev2	CAAGGAAACCCTGGACTACTGCGCCCTA	
Bcl11a-Wt-Fwd	CCATGACGGCTCTCCCACAAT	248
Bcl11a-Wt-Rev	GCGAGAATTCCCGTTTGCTT	
Bcl11a-Mut-Fwd	ACGAGTTCTTCTGAGGGGATC	420
Bcl11a-Mut-Rev	ATCCCGACTCCAGACTGGGAC	
Bcl11a-cko-Fwd	TAGCTCCTGCTAGCCAGGTTTCTT	377 (Wt);
Bcl11a-cko-Rev	CGAGGCTTGCAGAAACAGAAAGAT	470 (Cko);
Bcl11a-cko-Del	CTCGAAGGGAGGTTTCGGTATTGTG	700 (Del)
Bcl11b-lacZ-Fwd	GACACCAAACCGCTCTTTTAGACTA	644 (Wt); 527 (Mut)
Bcl11b-lacZ-Rev1	CATTGTGTAAGAGACAGGGTATAAG	
Bcl11b-lacZ-Rev2	CAAGGAAACCCTGGACTACTGCGCCCTA	
Bcl11b-Wt-Fwd	GCGTTTTCATCATTTACTTTGTCC	500
Bcl11b-Wt-Rev	TGGAAAAGATTCTCGGGGTCC	
Bcl11b-Mut-Fwd	GCCATTGTCACTGTCTCCCATC	500
Bcl11b-Mut-Rev	CATTGTTAGTCTCTTCTTTCCCGC	
Bcl11b-cko-Fwd	TGAGTCAATAAACCTGGGCGAC	243 (Wt);
Bcl11b-cko-Rev	GGAATCCTTGGAGTCACTTGTGC	345 (Cko);
Bcl11b-cko-Del	TCCTGGTAACACACAATTGC	450 (Del)
Rosa26-ERT-Cre-Fwd	TGTGGACAGAGGAGCCATAAC	500 (Wt); 300 (Mut)
Rosa26-ERT-Cre-Rev1	CATCACTCGTTGCATCGACC	
Rosa26-ERT-Cre-Rev2	AAGACCCAACCAACAGCAG	
BLG-Cre-Fwd	TCGTGCTTCTGAGCTCTGCAG	280
BLG-Cre-Rev	GCTTCTGGGGTCTACCAGGAA	
BAC Validation primers	**Primer sequence (5' – 3')**	**Size (bp)**
SpeI-Evi9-ext4-Ret-3'-2	TCTACTAGTCTCACCACCTGTACAGTAAGT	618
H3-Evi9-ext4-Ret-3'-1	GTCAAGCTTTGAGCCAGATCGAGCTAGGTC	
NotI-Ctip2-Ret-5'-1	ATATGCGGCCGCTTCCAGTAAGATGTGTCT	371
H3-Ctip2-Ret-5'-2	ATGTAAGCTTCTGGCTCACTTCGCGTGGTTA	
Bsd/Neo Validation primers	**Primer sequence (5' – 3')**	**Size (bp)**
Bcl11a-Bsd-PCR-F	TCTAGCCTCACATAGGGGAGAAAGT	420 (Wt)
Bcl11a-Bsd-PCR-R	CAGTTCAGCTAGTCAACTCTTTGGC	947 (Bsd); 7,251 (lacZ)
Bcl11a-Neo-PCR-F	GGCTGTTTCGCCTAAAGTTTTATTT	454 (Wt)
Bcl11a-Neo-PCR-R	CACAGACATAGATACTGCTGCTCTT	2,406 (Mut)
Bcl11b-Bsd-PCR-F	GCATTCCTGGGGACTGGGCTTGAGT	437 (Wt)
Bcl11b-Bsd-PCR-R	CGTCCCCTGTAGGTGTTCTTATTTT	964 (Bsd); 7,268 (lacZ)
Bcl11b-Neo-PCR-F	CAGAGGCGTCTCTGTGTGGAATTCA	498 (Wt)
Bcl11b-Neo-PCR-R	CCTTTGCCCTACAGTGCCCATCAGC	2,450 (Mut)

2.5.5 Transfection

Transfection was carried out using Lipofectamine LTX reagent (Invitrogen) according to manufacturer's protocol. Briefly, one day prior to transfection, KIM-2 cells were plated in 500 µl of growth medium so that cells would be 50-80% confluent on the day of transfection. 500 ng of plasmid DNA (MSCV-egfp; MSCV-Bcl11a-egfp and MSCV-Bcl11b-egfp) was diluted in 100 µl of Opti-MEM I reduced serum medium without serum and 0.5 µl of PLUS reagent (Invitrogen) was added and incubated at room temperature for 5 min. 1.25 µl of Lipofectamine LTX reagent was added directly to the DNA and the mixture was incubated at room temperature for 30 min. 100 µl of the DNA-Lipofectamine LTX complexes was added to each well of KIM-2 cells and cells were incubated at 37^{o}C/5% CO2 for 48 hours. GFP positive cells were then sorted out and RNA was extracted using PicoPure kit (as detailed below in Chapter 2.6.1).

2.6 RNA methods

2.6.1 Extraction of total RNA from cells

RNA from sorted cells was extracted using the PicoPure RNA extraction kit according to manufacturer instructions (Arcturus). Cells were pelleted by centrifugation at 5,000 G for 5 min and the supernatant was removed carefully. 100 µl of Extraction Buffer was added and cell mixture was incubated at 42^{o}C for 30 min. After centrifugation at 3,000 G for 2 min, the supernatant was removed and transferred to RNAse-free tubes. RNA purification columns were pre-conditioned by adding 250 µl of Conditioning Buffer to the membrane and incubating the columns at room temperature for 5 min before centrifugation at 16,000 G for 1 min. 100 µl of 70% ethanol was added into the cell extract and mixed well by pipetting up and down. The cell extracts were then pipetted onto the pre-conditioned RNA purification columns and centrifuged at 100 G to bind the RNA to the membrane. This was immediately followed by centrifugation at 16,000 G for 30 sec to remove the flow through. 50 µl of Wash Buffer 1 (W1) was pipetted into the purification columns and columns were spun at 8,000 G for 1 min. Next, 2.5 µl of DNase I stock solution in 10 µl of RDD buffer (Qiagen) was added to the purification column membrane and the columns were incubated at room temperature for 15 min. 50 µl of W1 was then added to the columns and columns were spun at 8,000 G for 1 min. The

columns were washed with 100 µl of Wash Buffer 2 before elution with 22 µl of Elution Buffer.

2.6.2 Extraction of total RNA from tissues

Frozen tissues were ground in a mortar using a pestle under liquid nitrogen and the powder was transferred into RNAse-free tubes. 1 ml of Trizol (Invitrogen) was added to the homogenized tissues and the cell suspension was incubated at room temperature for 5 min. 200 µl of chloroform was added to separate out the phenol phase (includes the genomic DNA). After centrifugation at 12,000 G for 15 min at 4^{o}C, the aqueous phase was removed and transferred to RNAse-free tubes. RNA was then precipitated by adding 250 µl of isopropyl alcohol and centrifugation at 12,000 G for 10 min at 4^{o}C. The RNA pellet was washed once with 1 ml of 75% ethanol with centrifugation at 7,400 G and air-dried before being re-suspended in an appropriate volume of RNAse-free water.

2.6.3 First strand cDNA synthesis

RNA quality and quantity was verified using a Nanodrop ND-100 Spectrophotometer (Thermo Scientific). Typically, 1-2 µg of RNA was made up to 11 µl with dd$H_2$0 and incubated with 1 µl of oligo-dT at 70^{o}C for 10 min and then placed on ice. 4 µl of first strand reverse transcription buffer, 2 µl of 0.1 M DTT and 1 µl of dNTPs were added and the mixture was incubated at 42^{o}C for 2 min. 1 µl of Superscript II Reverse Transcriptase was then added and the mixture was incubated at 42^{o}C for 1 hour. 1 µl of RNase H (Roche) was added and the mixture was incubated at 37^{o}C for 30 min to complete the first strand synthesis reaction. The samples were diluted using dd$H_2$0 and a PCR using the β-actin/Cyclophilin A primers was always performed to check for cDNA successful synthesis and concentration. All reagents were purchased from Invitrogen unless otherwise stated.

2.6.4 Semi-quantitative Real Time PCR

To investigate changes in expression of certain genes, semi-quantitative Reverse Transcription PCR (RT-PCR) was performed on cDNA using the primers listed in Table 2.4. PCR amplification was carried out using Extensor Hi-Fidelity PCR Master Mix 2

(2X, ABgene). 5 µl of the master mix was added to 1 µl of template (1-5 ng), 1 µl of each primer (10 µM) and 2 µl of PCR-grade water. PCR was performed using PTC-225 PCR machine (Peltier Thermal Cycler) with the following settings: 94^{o}C for 4 min, this was followed by 35 cycles of 94^{o}C for 30 sec, annealing temperature (See table 2.4) for 30 sec and 68^{o}C for 45 sec. This was then followed by 68^{o}C for 10 min. The PCR reaction was mixed with 6X loading dye and visualised on a 1.5% agarose gel.

Table 2.4. List of RT-PCR primers used.

RT-PCR primers	Primer Sequence (5'-3')	Annealing Temperature (^{o}C)	Cycles
α-casein-Fwd	CATCATCCAAGACTGAGCCAG	60	25
α-casein-Rev	CCTGTGGAAAGTAAGCCCAAAG		
β-casein-Fwd	GGCACAGGTTGTTCAGGCTT	60	25
β-casein-Rev	AAGGAAGGGTGCTACTTGCTG		
Whey acidic protein-Fwd	GCAGCATTTCATGTTGCCA	60	25
Whey acidic protein-Rev	TCGTTCTTGGCCTGCTGG		
α–lactalbumin-Fwd	TCGTTCCTTTGTTCCTGGTG	60	25
α-lactalbumin-Rev	GGGCTTGTAGGCTTTCCAGT		
Prolactin-Fwd	GTGGCCCCAATTCCTGTTTCTTTA	58	30
Prolactin-Rev	ATTTCTCCTGGCCCCATCTACTCC		
Prolactin-Receptor-Fwd	CACACGCGCAGATCTCCTTACC	58	30
Prolactin-Receptor-Rev	CGCTGTGTTCTGGGCTCGTG		
Gata3-Fwd	TGGGTGGGGCCTCATCCTCAG	60	30
Gata3-Rev	ACCGGGTCCCCATTAGCGTTCCT		
Stat6-Fwd	CTCTGTGGGGCCTAATTTCCA	60	30
Stat6-Rev	CATCTGAACCGACCAGGAACT		
Stat5-Fwd	CGCCAGATGCAAGTGTTGTAT	58	30
Stat5-Rev	TCCTGGGGATTATCCAAGTCAAT		
Stat3-Fwd	CAATACCATTGACCTGCCGAT	58	30
Stat3-Rev	GAGCGACTCAAACTGCCCT		
Akt1-Fwd	ATGAACGACGTAGCCATTGTG	61	30
Akt1-Rev	TTGTAGCCAATAAAGGTGCCAT		
Akt2-Fwd	ACGTGGTGAATACATCAAGACC	61	30
Akt2-Rev	GCTACAGAGAAATTGTTCAGGGG		
Notch1-Fwd	CAGCATGGCCAGCTCTGGTT	58	30
Notch1-Rev	AGCAGCATCCACATTGTTCA		
Notch2-Fwd	CATTGACGAGTGCACTGAGAGCTCCTGC	61	30
Notch2-Rev	AGTGCTGGCACAAGTGTTCAACAGGT		
Notch3-Fwd	ACACTGGGAGTTCTCTGTGAGATCA	60	30
Notch3-Rev	GTCTGCTGGCATGGGATACCCACTG		
Notch4-Fwd	CAGAACGTGGATCCCCTCAAGTTGCCTG	61	30
Notch4-Rev	GGCAGAGAGAGGGCAAGGAGTCATCAGC		
Jagged1-Fwd	CTGAGTCTTCTGCTCGCCCT	60	30
Jagged1-Rev	CGGCTAGGGTTTATCATGCC		
Jagged2-Fwd	CTGTGCAGCGTGTTCAGTG	61	35
Jagged2-Rev	GTGTCCACCATACGCAGATAAC		
Delta-like-ligand1-For	ACCGCAACTGCTGCCGCGGG	61	30
Delta-like-ligand1-Rev	GGCCGCTACTGTGAAGGTCC		
Delta-like-ligand3-For	CTGGTGTCTTCGAGCTACAAAT	61	35
Delta-like-ligand3-Rev	TGCTCCGTATAGACCGGGAC		
Delta-like-ligand4-For	TTCCAGGCAACCTTCTCCGA	62	35
Delta-like-ligand4-Rev	ACTGCCGCTATTCTTGTCCC		

Hes1-Fwd	ATAGCTCCCGGCATTCCAAG	61	30
Hes1-Rev	GCGCGGTATTTCCCCAACA		
Hes2-Fwd	CTGAAGGGTCTCGTATTGCCG	62	35
Hes2-Rev	CGCAGGTGCTCTAGTAGGC		
Hes3-Fwd	GCACGCATCAACGTGTCAC	61	35
Hes3-Rev	TGAGTTCTGGAGGCTTCTCAT		
Hes5-Fwd	AGTCCCAAGGAGAAAAACCGA	61	35
Hes5-Rev	GCTGTGTTTCAGGTAGCTGAC		
Hes6-Fwd	ACCACCTGCTAGAATCCATGC	61	30
Hes6-Rev	GCACCCGGTTTAGTTCAGC		
Hes7-Fwd	CGGGAGCGAGCTGAGAATAG	61	35
Hes7-Rev	CACGGCGAACTCCAGTATCT		
Elf5-For	ATGTTGGACTCCGTAACCCAT	61	20
Elf5-Rev	GCAGGGTAGTAGTCTTCATTGCT		
p63-Fwd	AGATCCCTGAACAGTTCCGAC	60	30
p63-Rev	CGACGAGAATCCATGTCAAAGTT		
NKCC1-Fwd	GGTTCTCCAAACTCACGG	60	25
NKCC1-Rev	GTCTTGCCATCCTCTTCCTC		
Muc1-Fwd	GGCATTCGGGCTCCTTTCTT	60	25
Muc1-Rev	TGGAGTGGTAGTCGATGCTAAG		
CK18-Fwd	CAGCCAGCGTCTATGCAGG	58	25
CK18-Rev	CTTTCTCGGTCTGGATTCCAC		
CK14-Fwd	TGAGAGCCTCAAGGAGGAGC	58	30
CK14-Rev	TCTCCACATTGACGTCTCCAC		
Smooth muscle actin-α-Fwd	AGATTGTCCGTGACATCAAGG	60	25
Smooth muscle actin-α-Rev	TTGTGTGCTAGAGGCAGAGC		
LIF-Fwd	GCTATGTGCGCCTAACATGAC	63	35
LIF-Rev	CGCTCAGGTATGCGACCAT		
ERα-Fwd	CCTCCCGCCTTCTACAGGT	63	30
ERα-Rev	CACACGGCACAGTAGCGAG		
Bcl11a-Fwd	TGGTATCCCTTCAGGACTAGGT	61	35
Bcl11a-Rev	TCCAAGTGATGTCTCGGTGGT		
Bcl11b-Fwd	CCCGACCCTGATCTACTCAC	61	35
Bcl11b-Rev	CTCCTGCTTGGGACAGATGCC		
Cyclophilin A-Fwd	CCTTGGGCCGCGTCTCCTT	55	25
Cyclophilin A-Rev	CACCCTGGCACATGAATGGTG		
β-actin-Fwd	GTGGGCCGCTCTAGGCACCAA	58	25
β-actin-Rev	CTCTTTGATGTCACGCACGA		
p85α-Fwd	GCCCCGTGCTTTTCAGATTTC	60	30
p85α-Rev	TCCTGCTGGTATTTGGACACTGGGTAG		
p55α-Fwd	GTTACAGTGCGGGCCGTATAGGTTTTA	60	30
p55α-Rev	TCCTGCTGGTATTTGGACACTGGGTAG		
p50α-Fwd	CTGGCAGTTCAAAGCGAAACCGT	60	30
p55α-Rev	TCCTGCTGGTATTTGGACACTGGGTAG		

2.6.5 Quantitative Real Time PCR

2.6.5.1 TaqMan

Quantitative real-time TaqMan PCR was used for detection of deletion efficiency in genomic DNA following Cre recombinase expression. The PCR was carried out with the addition of 12.5 µl of ABsolute QPCR mix (Thermo) with 1 µl of each primer (10 µM) and 0.5 µl of probes (0.25 µM; MWG, Ebersberg, Germany). The real-time PCR reactions were run in an ABI PRISM 7900HT (Applied Biosystems) in triplicate. Probes

were labelled with reported dye (FAM) at the 5' end and the quencher dye TAMRA at the 3' end. Primers and probes used are listed in Table 2.5.

2.6.5.2 SYBR Green

Real time PCR was performed to quantify the relative amounts of mRNA expression of gene of interest against *Cyclophilin* A as an internal control. Bio-Rad iCycler PCR (Bio-Rad) machine with a Halogen Tungsten lamp were used and data was analysed using iCycler iQ software (Bio-Rad). The PCR was performed using iQ Supermix (Bio-Rad) which contains the Taq enzyme, buffer and dNTPs. SYBR Green I (Bio-Rad) DNA fluorescent dye was used with high affinity to dsDNA and an excitation wavelength of 488 nm. Primers used are listed in Table 2.5

Table 2.5. List of qRT-PCR primers used.

qRT-PCR (TaqMan)	Primer Sequence (5'-3')
Bcl11a-Fwd	AAAGGCACTGATGAAGATATTTTCTCT
Bcl11a-Rev	CGACGGCTCGGTTCACAT
Bcl11a-Probe	TCTCCTTCTTTCTAACCCGGCTCTCCC
Bcl11b-Fwd	AGTGCCTTCGACCGAGTCAT
Bcl11b-Rev	GAAGTCCATGGCAGGAGAGTCT
Bcl11b-Probe	CGCCTGAACCCCATGGCCA
β-actin-Fwd	TTCAACACCCCAGCCATGTA
β-actin-Rev	TGTGGTACGACCAGAGGCATAC
β-actin-Probe	TAGCCATCCAGGCTGTGCTGTCCC
qRT-PCR (SYBR-Green)	**Primer Sequence (5'-3')**
Bcl11a-Fwd	TGGTATCCCTTCAGGACTAGGT
Bcl11a-Rev	TCCAAGTGATGTCTCGGTGGT
Bcl11b-Fwd	CCCGACCCTGATCTACTCAC
Bcl11b-Rev	CTCCTGCTTGGGACAGATGCC

2.6.6 *In situ* hybridization

2.6.6.1 Cloning and synthesis of probes

Primers were used to PCR amplify probes from a mouse embryo 10.5 days post-coitum total cDNA library constructed as detailed in Chapter 2.6.3. PCR products were then cloned into either pCR-II-TOPO or pCR-Blunt-II-TOPO vectors (Invitrogen) and transformed into TOP10 cells (Invitrogen). Transformants were selected on LB agar containing either Amp (pCR-II-TOPO) or Kan (pCR-Blunt-II-TOPO). 8 clones were picked for each probe and verified using restriction digestion and sequencing to

determine the orientation of the insert in the vector backbone. Appropriate enzymes were then chosen to linearize the plasmids to enable synthesis of the antisense strand by *in vitro* transcription. 2 µg of plasmid was digested with 1 µl of appropriate enzyme with 2 µl of buffer (NEB) in a total volume of 20 µl. Linearized plasmids were then purified using a mini-preparation spin column (Qiagen) and eluted in 20 µl of ddH_2O. 1 µg of linearized probe was then incubated at 37°C for 2 hours with 2 µl of transcription buffer (10X), 2 µl of NTP labelling mixture, 1 µl of RNase inhibitor, and 2 µl of SP6 or T7 RNA polymerase. After *in vitro* synthesis, 2 µl of DNase I was added and the mixture was incubated at 37°C for 15 min before purifying the probes using ProbeQuant G-50 Micro columns (GE Healthcare). Reagents were obtained from Roche unless otherwise stated.

Table 2.6. List of primers used for cloning *in situ* probes.

In situ Probe	Primer sequence (5' – 3')	Size (bp)	Vector	Linearization Enzyme	Polymerase
Fgf10-probe-Fwd	AATGTGGAAATGGATACTGACACAT	631	pCRII-TOPO	*Not* I	SP6
Fgf10-probe-Rev	CTATGTTTGGATCGTCATGGGGAGG				
Fgfr2TK-probe-Fwd	ACAGTTCTGCCAGCGCCTGTGAGAG	949	pCRII-TOPO	*Not* I	SP6
Fgfr2TK-probe-Rev	CTTCAGGAGCCATCCACTTGACTGG				
Lef1-probe-Fwd	TCATCTTTGGTTAACGAGTCCGAAA	359	pCR-Blunt II-TOPO	*Hind* III	T7
Lef1-probe-Rev	ATGTGTGACGGGTGGGATCCCGGAG				
Notch1-probe-Fwd	CAGCATGGCCAGCTCTGGTT	846	pCR-Blunt II-TOPO	*Xho* I	SP6
Notch1-probe-Rev	AGCAGCATCCACATTGTTCA				
Wnt10b-probe-Fwd	GGAGGGCAGCGCCAGAGTTCC	257	pCR-Blunt II-TOPO	*Bam* HI	T7
Wnt10b-probe-Rev	AGGCTGCCACAGCCATCCAAGAGG				
Tbx3-probe-Fwd	GAGATGGTCATCACGAAGTC	567	pCRII-TOPO	*Not* I	SP6
Tbx3-probe-Rev	CTGCAATGCCCAATGTCTCG				

2.6.6.2 Whole mount hybridization

Embryos were dissected from time mated females and staged before fixing them in 4% paraformaldehyde (PFA) at 4°C for 48 hours. The embryos were then washed sequentially in PBST (PBS with 0.1% Tween-20), 25%, 50%, 75% Methanol (MeOH)/PBST and 100% MeOH for 5 min each at room temperature (with shaking). Embryos were rehydrated by sequential washes in 75%, 50%, 25% MeOH/PBST and PBST for 5 min at room temperature (with shaking). Next, embryos were bleached using 6% hydrogen peroxide (in PBST) for 1 hour at room temperature, washed thrice with PBST before treatment with 10 µg/ml of Proteinase K (in PBST) for 15 min at room temperature. Subsequently, embryos were washed thrice with PBST, post-fixed with 4%

PFA/0.2% Glutaldehyde/PBST for 20 min at room temperature and washed twice with PBST prior to incubating them in hybridization solution (50% formamide, 5X SSC, 1% SDS, 50 µg/ml tRNA, 50 µg/ml Heparin) for 1 hour at 70°C. Finally, embryos were incubated overnight at 70°C in preheated hybridization solution containing 1 µg/ml of antisense riboprobe. On the second day, the embryos were washed twice with preheated solution 1 (50% formamide, 5X SSC, 1% SDS) at 70°C for 30 min each, thrice with preheated solution 2 (50% formamide, 2X SSC) at 65°C for 30 min each and thrice with TBST (1X; 8 g of NaCl, 25 ml of Tris-HCl, pH 7.5, 0.1% of Tween-20). The embryos were then pre-blocked with 10% sheep serum (in TBST) for 90 min before incubating with anti-DIG antibody (Roche) overnight at 4°C. On the third day, the embryos were washed five times with TBST (with 50 mg/ml of Levamisole, Sigma) for 1 hour each (with shaking) at room temperature. Embryos were left overnight in TBST at room temperature to improve background. To develop the staining, embryos were incubated in NBT/BCIP solution (Roche) at room temperature in the dark until desired reaction solution was achieved. Reaction was stopped by washing the embryos in TE (pH 8) and fixing in 4% PFA for 1 hour at 4°C. Embryos were then imaged using a LEICA MZ75 light microscope.

2.7 Protein methods

2.7.1 Protein extraction

Frozen tissues were ground in a mortar using a pestle under liquid nitrogen and the powder was transferred into a 1.5 ml tubes. Tissue powder was re-suspended in 100-300 µl of RIPA buffer [50 mM Tris (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1X Cocktail protease inhibitors (Roche), 1 mM Na_3VO_4, and 1 mM NaF] and transferred to a 1.5 ml tube. The lysate was mechanically ground using a micropestle (Eppendorf) and then passed through a syringe 5-8 times and left on ice for 40 min. Lysate was spun for 15 min at 16,000 G using a bench-top centrifuge. The supernatant was collected and frozen. Also, 10 µl of the supernatant was collected in a separate tube for BCA protein concentration assay.

2.7.2 BioCinchominic Acid (BCA) protein concentration assay (Pierce)

Bovine Serum Albumin (BSA) (Sigma) was made at required concentrations of 0.1 mg, 0.2 mg, 0.5 mg, 1.0 mg and 2.0 mg which were used as standards for the assay. The BCA reagents were mixed according to manufacturer's protocol in a ratio of 50:1 (A:B) respectively. 200 ul of the reagent mix was added to 5 ul of standard or sample in a 96 well plate. Each standard or sample was measured in triplicate. The sample were diluted in water prior to usage in the assay, the dilution factor depended on how concentrated the sample was. The range of dilution was between 1:4 and 1:16. This step is crucial for accuracy of the results. The reaction was incubated at room temperature for 20 min before the light absorbance was measured at a wavelength of 570 nm using a DYNATECH-MR5000 plate reader. The read out from the standards less the background, was used to plot a graph and the slope of which was used to calculate the concentration of the samples.

2.7.3 SDS-PAGE

The protein samples were made up to a final concentration of 30 µg using RIPA buffer and Loading Buffer [125 mM Tris-HCl (pH 6.8), 2.5% SDS, 20% glycerol, 0.002% Bromophenol blue and 5% β-mercaptoethanol]. The samples were boiled at 95^{o}C for 5 min and then were kept on ice. SDS-PAGE Criterion precast Tris-HCl resolving gels (Biorad) were used. Samples were loaded and gels were run at 100 V for 90-120 min at room temperature using Biorad powepac 300. 1X Running buffer (3.03 g Tris, 14.4 g Glycine, 1 g SDS and ddH_2O to 1 L).

2.7.4 Immunoblotting

Proteins were immunoblotted onto PVDF membranes (Millipore-immobilon FL) using the Biorad wet transfer tanks. The gels were placed in a cassette facing the PVDF membrane and electric current was applied at 400 mA for 1 hour. The transfer buffer used varied depending on the size of protein of interest. For high molecular weight proteins, low percentage methanol was used while for low molecular weight proteins

higher percentage of methanol was used. 1X Transfer buffer (192 mM Glycine, 25 mM Tris, 0.1% SDS, 5-20% v/v 100% Methanol, pH 8.3).

2.7.5 Primary antibody incubation

The PVDF membranes were incubated in blocking solution for 1 hour at room temperature to reduce background signal in the consequent steps. The blocking buffer and dilution differs between antibodies used are summarised in Table 2.7. All primary antibodies were incubated overnight at 4^{o}C whilst shaking.

Table 2.7. List of antibodies used for Western blot analysis.

Antibody	Clone	Source	Dilution	Blocking Buffer	Species
Bcl11a	BL1796	Bethyl Laboratories	1:1000	5% Milk	Rabbit
Stat5	9352	Cell Signalling	1:1000	5% Milk	Mouse
pStat5A/B (phospho Tyr)	9351	Cell Signalling	1:1000	5% Milk	Rabbit
Stat3	9139	Cell Signalling	1:1000	5% Milk	Mouse
pStat3 (phospho Tyr)	9131	Cell Signalling	1:1000	5% Milk	Rabbit
Stat6	M-20	Santa Cruz	1:1000	5% Milk	Rabbit
pStat6 (phospho Tyr)	177C322	Abcam	1:8000	5% BSA	Mouse
Gata-3	H-48	Santa Cruz	1:1000	5% Milk	Rabbit
Notch1	C-20	Santa Cruz	1:1000	5% Milk	Rabbit
Notch3	M-134	Santa Cruz	1:1000	5% Milk	Rabbit
Jagged1	H114	Santa Cruz	1:1000	5% Milk	Rabbit
Pan-p85	N-SH2	Millipore	1:1000	5% Milk	Rabbit
Akt	9272	Cell Signalling	1:1000	5% Milk	Rabbit
pAkt (phosphor Ser 473)	4058	Cell Signalling	1:1000	5% Milk	Rabbit
WAP	M-16	Santa Cruz	1:1000	5% BSA	Goat
β-casein	-	Gift from Bertr Binas	1:1000	5% BSA	Goat
Cleaved caspase 3	9664	Cell Signalling	1:1000	5% Milk	Rabbit
β-actin	N-21	Santa Cruz	1:1000	5% Milk	Rabbit

2.7.6 Secondary antibody incubation and detection

The primary antibodies were removed and the membranes were rinsed once with 1X PBS-0.1%Tween (Sigma) (PBST). The appropriate secondary antibody was diluted to a final concentration of 1:2000 in the blocking buffer and incubated with the membrane for 45 min. The secondary antibodies used were all conjugated with horseradish peroxidase (HRP) (DAKO). The membrane was then washed thrice with 1X PBST for 5 min each (or TBST in case of pAKT and pStat6) to get rid of non-specific binding. ECL or ECL-plus (Amersham) was used according to the manufacture's instructions as a substrate for the HRP enzyme. The chemi-luminescence was detected using Hyper-film

(Amersham). All western blots were scanned and edited using Jasc Paint Shop Pro software.

2.7.7 Stripping PVDF membranes

To allow the probing of a single membrane with multiple antibodies, the previous primary antibody has to be striped off. The membrane was incubated with 25 ml of strip buffer and 175 µl of 2-Mercaptoethanol for 1 hour at 50°C. The membrane was then washed thoroughly using ddH_2O, appropriate blocking buffer applied and incubated with primary antibody overnight. Strip buffer (40 g SDS, 15 g Tris, ddH_2O to 2 L, pH 6.8).

2.8 Whole mount X-gal staining

2.8.1 Embryo

Embryos were removed and dissected with the yolk sac and placenta intact before fixation with 4% PFA overnight at 4°C with shaking. Fixation time (7.5-11.5 dpc: 15 min; 12.5-14.5 dpc: 30 min; 15.5-17.5 dpc: 60 min). Embryos were then washed thrice with ice-cold PBS (pH 8) for 20 min each at 4°C with shaking before staining in X-gal staining solution [2mM $MgCl_2.6H_2O$, 0.01% deoxycholic acid, 0.02% IGEPAL CA-630, 5 mM potassium ferrocyanide ($K_4Fe(CN)_6.3H_2O$), 5 mM potassium ferricyanide ($K_3Fe(CN)_6$), 1 mg/ml X-gal in dimethylformamide, PBS pH 8] at 4°C with shaking for up to 48 hours. Stained embryos were then washed briefly in PBS (pH 8) before post-fixing in 4% PFA overnight at 4°C with shaking. Embryos were then cleared with 50% glycerol (in PBS, pH 7.4) overnight at 4°C with shaking and stored in 70% glycerol (in PBS, pH 7.4, 0.01% sodium azide) at room temperature for a week prior to imaging.

2.8.2 Adult tissues

Adult mice were anesthetized with isofluorane and a midline incision was made to visualize the heart and the great vessels. The abdominal aorta was cannulated with a 30-gauge needle coupled to PE-10 tubing to allow aortic retrograde perfusion of 4% PFA. Following perfusion, various tissues such as the brain, spinal cord and the internal organs were dissected out and fixed in 4% PFA for 1 hour. Tissues were then washed thrice with ice-cold PBS (pH 8) at 4°C with shaking before staining in X-gal staining solution [2mM

$MgCl_2.6H_2O$, 0.01% deoxycholic acid, 0.02% IGEPAL CA-630, 5 mM potassium ferrocyanide ($K_4Fe(CN)_6.3H_2O$), 5 mM potassium ferricyanide ($K_3Fe(CN)_6$), 1 mg/ml X-gal in dimethylformamide, PBS pH 8) at 4°C with shaking for up to 48 hours. Stained tissues were then washed briefly in PBS (pH 8) before post-fixing in 4% PFA overnight at 4°C with shaking. Tissues were then cleared with 50% glycerol (in PBS, pH 7.4) overnight at 4°C with shaking and stored in 70% glycerol (in PBS pH 7.4, 0.01% sodium azide) at room temperature for a week prior to imaging.

2.8.3 Mammary gland

Mammary glands were dissected out from female mice and spread onto a clean glass slide before fixing in 4% PFA for 1 hour at 4°C. Glands were then rinsed briefly in PBS (pH 8) and washed thrice with wash buffer (PBS with 2 mM $MgCl_2$) for 5 min each at room temperature with shaking. Glands were then incubated in permeabilization solution (PBS with 0.2% NP-40, 0.01% deoxycholic acid, 2 mM $MgCl_2$) for 1 hour at room temperature with shaking. Next, glands were incubated in X-gal mixer [25 mM potassium ferrocyanide ($K_4Fe(CN)_6.3H_2O$), 25 mM potassium ferricyanide ($K_3Fe(CN)_6$), 0.2% NP-40, 0.01% deoxycholic acid, 2 mM $MgCl_2$, PBS] for 1.5 hours at 37°C. 1 mg/ml of X-gal (Invitrogen) was then added to the X-gal mixer and glands were stained for 1-3 days at 37°C. After staining, glands were post-fixed in 4% PFA at 4°C overnight and then processed through sequential 1 hour washes of acetone, 70% ethanol, 95% ethanol, 100% ethanol followed by overnight clearing in xylene. Glands were then imaged prior to being embedded in paraffin for sectioning.

2.9 Histology and immunohistochemistry

2.9.1 Tissue carnoys fixative and whole mount carmine alum staining

Mammary glands were dissected out and spread out onto a glass slide and incubated in carnoys fixative (6 parts 100% ethanol, 3 parts chloroform, 1 part glacial acetic acid) overnight. The slide was then placed in carmine alum stain (1 g carmine, 2.5 g aluminium potassium sulphate and ddH_2O) overnight. The slide was then washed with

ethanol and cleared in xylene for 1 day. After photographic documentation the slide was stored in xylene.

2.9.2 Paraffin sections and Hematoxylin and Eosin (H&E) staining

Mammary glands were collected as described earlier. Glands were fixed in 4% PFA for 24 hours at 4^{o}C. The glands were then transferred into 70% ethanol and stored at -20^{o}C till sectioning. All tissues were embedded in wax and sectioned at 5 µm thickness on a glass slide. The sections were then de-waxed using three 5 min xylene washes and re-hydrated in a decreasing alcohol series and rinsed in ddH_2O before staining in Hematoxylin solution for 5 min and placed under running tap water till it turned blue. The slides were then placed in 1% aqueous Eosin for 1 min and then washed in running tap water.

2.9.3 Fluorescence immunohistochemistry

Paraffin embedded sections were de-waxed in three 5 min xylene washes and re-hydrated using 5 min washes in serial dilutions of ethanol/H_2O/PBS in the following order 100%, 100%, 95%, 95%, 50%, H_20, H_20, PBS, PBS (0.1% Triton X100) and PBS. The sections were then boiled in 10 mM Tri-Sodium Citrate Buffer (pH 6) using a steam cooker under pressure for 12 min. The sections were left in the cooling buffer for 20 min, washed once in PBS for 5 min and then dried before drawing wax barriers around the sections using a pap pen (Vector laboratories). Blocking solution (10% goat serum in PBS) was then applied on the tissue sections and incubated in a humidified chamber for 1 hour at room temperature. Following one rinse in PBS, the sections were then either incubated with primary antibody or blocking buffer overnight at 4^{o}C in a humidified chamber. Primary antibodies used are listed in Table 2.8. Following the overnight incubation with the primary antibody, the sections were washed thrice in PBS for 5 min each and incubated with either Alexa Fluor 488 (Invitrogen) or Cy3 (Sigma) conjugated secondary antibody diluted in blocking buffer and incubated in the humidified chamber in the dark at room temperature for 1 hour. The sections were then washed thrice with PBS for 5 min each and incubated with Bisbenzimide-Hoechest 33342 (Sigma) to stain the DNA for 5 min in the dark at room temperature. After a wash with PBS the sections were

mounted with 50% PBS-50% Glycerol solution and cover-slip was placed on top and sealed using clear nail polish (Worldwide glass resources Ltd). Pictures were taken immediately afterwards or within two days of mounting.

Table 2.8. List of antibodies used for immunohistochemistry.

Antibody	Clone	Source	Dilution	Species
pStat5A/B (phospho Tyr)	9351	Cell Signalling	1:100	Rabbit
pStat3 (phospho Tyr)	9131	Cell Signalling	1:100	Rabbit
Gata-3	HG3-31	Santa Cruz	1:100	Mouse
Notch1	C-20	Santa Cruz	1:100	Rabbit
Jagged1	H-114	Santa Cruz	1:100	Rabbit
β-casein	-	Gift from Bertr Binas	1:1000	Goat
Cleaved Caspase 3	9664	Cell Signalling	1:100	Rabbit
Aquaporin 5	178615	Calbiochem	1:50	Rabbit
Cytokeratin 14	LL002.	Abcam	1:100	Mouse
Cytokeratin 18	CY90	AbD Serotec	Single drop	Mouse
Smooth muscle actin	Ab-1	NeoMarkers	1:1000	Mouse
p63	4A4	Abcam	1:50	Mouse
Estrogen receptor-α	MC-20	Santa Cruz	1:50	Rabbit
Ki67	KLH	Abcam	1:50	Rabbit

2.10 FACS staining and analysis

2.10.1 Hematopoietic cells

8-12 weeks old adult mice were used. Bone marrow cells were flushed from the long bones (tibias and femurs) using a 26G syringe with PBS (without calcium or magnesium), supplemented with 1% heat-inactivated serum (GIBCO). Spleen or thymus were placed in PBS and gently homogenized with the end of a syringe. Cells were triturated and filtered through a 30 μm mesh to obtain single-cell suspension. Lysis of the red blood cells in ammonium chloride (NH_4Cl) was carried out before using cells for staining.

Prior to staining for hematopoietic stem cells in the bone marrow, depletion of mature hematopoietic cells such as T cells, B cells, monocytes/macrophages, granulocytes, and erythrocytes as well as their committed precursors from whole bone marrow cells using the Lineage cell depletion kit (Miltenyi Biotec). Cells were first stained with a cocktail of biotinylated antibodies against a panel of lineage antigens [CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4, and TER-119] and incubated on ice in the dark for 10 min. Anti-Biotin MicroBeads were then added and cells were incubated in the fridge at 4^{o}C for a further 15 min. 1 ml of MACS buffer (0.5% BSA, 5 mM EDTA

in PBS) was added and cells were spun at 375 G for 5 min. The supernatant was decanted and cells were re-suspended in 500 µl of MACS buffer and added to a pre-washed MS column (Miltenyi Biotec). The flow-through containing the lineage-negative cells was collected and used for staining with Sca1 and c-kit primary antibodies. Prior to staining for T cell progenitors in the thymus, CD4-positive and CD8-positive thymocytes were removed using CD4 (L3T4) and CD8 (Ly2) depletion kit (Miltenyi Biotec). Cells were first stained with biotinylated anti-CD4 and anti-CD8 antibodies and incubated on ice in the dark for 10 min. Anti-Biotin MicroBeads were then added and cells were incubated in the fridge at 4^{o}C for a further 15 min. 1 ml of MACS buffer was added and cells were spun at 375 G for 5 min. The supernatant was decanted and cells were re-suspended in 500 µl of MACS buffer and added to a pre-washed MS column (Miltenyi Biotec). The flow-through containing the CD4 and CD8-negative thymocytes was collected and used for staining with CD44 and CD25 primary antibodies.

Primary antibodies used are listed in Table 2.9. Cells were stained on ice in the dark for 20 min. 1 ml of PBS (with 1% FCS) was then added and cells were centrifuged at 375 G for 5 min. Supernatant was then decanted and cells were re-suspended in 500 µl of PBS (with 1% FCS) before analysis. FACS analysis was done using CyAN ADP (DakoCytomation) or MoFlo (DakoCytomation) and gates were set to exclude >99.9% of cells labelled with isoform-matched control antibodies conjugated with the corresponding fluorochromes. Three mice of each genotype were analyzed.

2.10.2 Mammary cells

Mammary glands from 4-14-week-old virgin female mice were dissected and mammary epithelial cell suspensions were prepared as previously described (Stingl et al., 2006). Briefly, dissected mammary glands were digested for 8 hours at 37^{o}C in DMEM:F12 (Gibco) with collagenase (300 U/ml) and hyaluronidase (100 U/ml). After vortexing and lysis of the red blood cells in NH_4Cl, epithelial cell clumps were first treated by gentle pipetting for 2 min in 0.25% trypsin, and then for 2 min in dispase II (5 mg/ml) plus DNase I (0.1 mg/ml; Sigma) followed by filtration through a 30 µm mesh. Primary antibodies used are listed in Table 2.9. Secondary antibody used: Strepavidin-PE-Texas Red (PE-TR, Molecular Probes). Apoptotic cells were excluded by elimination

of propidium iodide (PI) positive cells. All antibody staining was carried out on ice for 10 min in dark. All reagents were from StemCell Technologies Inc. unless otherwise specified. FACS analysis was done using CyAN ADP (DakoCytomation) and all sorts were performed using MoFlo (DakoCytomation) and gates were set to exclude >99.9% of cells labelled with isoform-matched control antibodies conjugated with the corresponding fluorochromes.

2.10.3 FDG staining

Fluorescein di-β-D-galactopyranoside (FDG; Sigma), a fluorescent substrate for β-galactosidase, was used for flow cytometric analysis to determine expression of *Bcl11* in hematopoietic and mammary epithelial cells. Hematopoietic or mammary cells were first stained with primary and/or secondary antibodies as detailed above (Chapter 2.10.1 and 2.10.2) before being used for FDG staining. Cell samples and FDG stock (2 mM in DMSO) were pre-warmed at 37°C for 5 min. Next, an equal volume of FDG stock was added and the cell mixture was incubated at 37°C for 1 min before adding 2 ml of HBSS and incubating on ice for 1 hour in the dark. For staining of *Bcl11b*$^{lacZ/+}$ thymocytes, phenylethyl-β-d-thiogalactopyranoside (PETG, 200 mM; Invitrogen), an inhibitor to β-galactosidase, was added to the cell mixture after 10 min.

Table 2.9. List of antibodies used for FACS analysis.

Antibody	Fluorescent dye	Clone	Source	Dilution
B220	PE	RA3-6B2	BD Biosciences	1 μg/ml
CD19	PE	1D3	BD Biosciences	1 μg/ml
Gr1	PE	RB6-8C5	BD Biosciences	1 μg/ml
Ter119	PE	TER-119	BD Biosciences	1 μg/ml
CD4	PE	GK1.5	BD Biosciences	1 μg/ml
CD8	PE-Cy5	53-6.7	BD Biosciences	1 μg/ml
CD44	PE	IM7	BD Biosciences	1 μg/ml
CD25	APC	PC61	BD Biosciences	1 μg/ml
Sca1	PE	D7	BD Biosciences	1 μg/ml
c-kit	APC	2B8	BD Biosciences	1 μg/ml
CD45	Biotin	30-F11	eBioscience	1 μg/ml
Ter119	Biotin	TER-119	eBioscience	1 μg/ml
CD31	Biotin	390	eBioscience	1 μg/ml
CD24	PE	M1/69	eBioscience	1 μg/ml
CD49f	AF647	GoH3	eBioscience	1 μg/ml
CD49b	FITC	HMa2	eBioscience	1 μg/ml
Sca1	AF647	D7	eBioscience	1 μg/ml

2.11 Mammary colony-forming-cell (Ma-CFC) assay

For Ma-CFC assays, mammary epithelial cells were prepared and stained with CD24, CD49f and FDG as described above (Chapter 2.10.2). Next, lineage negative $CD24^{+}CD49f^{+}$ epithelial cells were sorted into FDG^{-} (3,000 cells) and FDG^{+} (500 cells) fractions using MoFlo (DakoCytomation). The freshly-sorted cells were then plated onto irradiated feeders (10,000 feeders per ml of media) in NSA media and maintained for a week at $37^{o}C/5\%$ CO_2 as described previously (Stingl et al., 2006). To terminate the assays, the plates were washed once with PBS and fixed in ice-cold acetone:methanol (1:1) for 5 min. Plates were then washed once with water and stained with Giemsa (Merck) for 5 min before a final rinse with water. The number of colonies was enumerated under a dissecting microscope. Composition of NSA media: 9 parts NeuroCult NSA base medium (Human) (StemCell Tech); 1 part NeuroCult NSA supplements (StemCell Tech); 1:100 dilution of N2 supplements (Gibco); 10 ng/ml basic fibroblast growth factor (Sigma); 10 ng/ml epidermal growth factor (Sigma); 10 ng/ml insulin (Sigma) and 5% FBS (StemCell Tech).