CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 REAGENTS

• PBS (phosphate buffered saline; PAA, cat. #: H15-002)

• BME (β-mercaptoethanol; Sigma-Aldrich, cat. #: M7522)

• Hypoxanthine-aminopterin-thymidine (HAT) supplement (Invitrogen, cat. #: 21060-01)

• Hypoxanthine-thymidine (HT) supplement (Invitrogen, cat. #: 41065-012)

• Trypsin (+ glucose)

0.1 g of ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, cat. #: E5134) and 0.5 g of D-glucose (Sigma-Aldrich, cat. #: G7528) were added to 500 ml of PBS (Invitrogen, cat. #: 14190094). The mixture was sterilised through a 0.2 µm syringe filter. Subsequently 5 ml of chicken serum (Invitrogen, cat. #: 16110082) and 10 ml of 2.5% trypsin were added to the mixture (Invitrogen, cat. #: 15090046) and aliquots were stored at -20ºC.

• GPS (glutamine/penicillin/streptomycin)

58.4 g of glutamine (Amresco, cat. #: 0374-500G), 10 g of streptomycin (Sigma-Aldrich, cat. #: S9137-100G) and 6 g of penicillin (Sigma-Aldrich,

cat. #: P3032-100MU) were added to 2 l of Milli-Q H_2O. The mixture was sterilised through a 0.2 µm syringe filter and aliquots were stored at -20ºC.

• G418 (125 mg/ml)

10 ml of PBS was added to 5 g of geneticin (Invitrogen, cat. #: 11811031). The mixture was diluted to a final volume of 30.8 ml, sterilised through a 0.2 µm syringe filter and aliquots were stored at -20ºC.

• 0.1% gelatin

25 ml of 2% gelatin solution (Sigma-Aldrich, cat. #: G1393) was added to 500 ml of PBS (1x; without magnesium chloride; without calcium chloride – Invitrogen cat #: 14190094).

• M15 medium (was used for no more then 3 weeks due to glutamine & BME lose potency)

90 ml of foetal bovine serum (FBS) (PAA, cat. #: A15-101), 6 ml of GPS and 6 ml of BME (100x stock) were added to 500 ml of high glucose Dulbecco's Modified Eagle Medium (DMEM) (PAA, cat. #: E15-011). If feeder cells were not used, leukaemia inhibiting factor (LIF) was added to M15 medium.

If selection was required:

- for G418:

  0.56 ml of stock G418 (125 mg/ml) was added to obtain a final concentration of 125 µg/ml,

  0.672 ml of stock G418 (125 mg/ml) was added to obtain a final concentration of 150 µg/ml,

  0.784 ml of stock G418 (125 mg/ml) was added to obtain a final concentration of 175 µg/ml,

  0.896 ml stock G418 (125 mg/ml) was added to obtain a final concentration of 200 µg/ml.

- for puromycin

  100 mg of puromycin (Sigma-Aldrich, cat. #: P8833) was added to 33.3 ml of PBS to obtain a final concentration of 3 mg/ml.

All components of M15 medium, except high glucose DMEM and FBS, were sterilised through a 0.2 µm syringe filter. High glucose DMEM was stored at +4°C, while all the components of M15 medium were stored at -20°C.

• M7 feeder culture medium

45 ml of FBS (aliquots were stored in -20°C) and 6 ml of GPS were added to 500 ml of knockout DMEM.

• Freeze medium (was always made fresh before use)

10% FBS and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich cat #: D2650-100ML) were added to 80% knockout DMEM and the mixture was sterilised through a 0.2 µm syringe filter.

• Hepes/M15 medium

1 ml of 1 M hepes buffer (Invitrogen, cat #: 15630049) was added to 49 ml of M15 medium.

2.2 METHODS

2.2.1 CELL CULTURE

2.2.1.1 CULTURE CONDITIONS OF ES CELL LINES

The feeder-free ES cell line, E14Tg2a (129P2/OlaHsd), was cultured as previously described (Smith 1991). The feeder-dependent ES cell line, AB2.2 (129S7/SvEvBrdHprt$^{b\text{-}m2}$), was cultured as previously described (Ramirez-Solis 1995), on the puromycin-resistant feeder cell line, MEF P-SNL 76/7-4. Both ES cell lines were cultured in M15 medium and maintained at 37°C with 5% CO_2, with the medium changed daily unless stated otherwise.

2.2.1.2 TRANSFECTION OF TARGETING VECTORS INTO ES CELLS BY ELECTROPORATION

Approximately 100 µg of a targeting vector's DNA was linearized with the appropriate restriction enzyme overnight. Next morning, the targeting vector's DNA was purified by ethanol precipitation. 2.5 times the volume of 100% ethanol was added to the tube containing the mixture of the vector and restriction enzyme, vortexed, and left on ice for 5 minutes. Then, the tube was centrifuged at 12,000 rpm for 1 minute and the ethanol was discarded. The pellet was washed twice with 70% ethanol and subsequently air-dried at room temperature. The air-dried targeting vector's DNA was re-suspended in 0.1 ml of PBS to a final concentration of 1 mg/ml. 15 µg of DNA was used for electroporation, unless stated otherwise. ES cell electroporation was performed as described previously (Ramirez-Solis 1993). 80% confluent ES cells were fed 2 – 3 hours before electroporation. Then, the medium was aspirated and the plate/flask washed twice with PBS. Next, 1x trypsin was added to the plate/flask according to **Table 2.1**, and the plate/flask was incubated at 37°C for 4 minutes. After incubation the M15 medium was added to the plate/flask according to **Table 2.1** to neutralize the trypsin. The cells were next centrifuged at 1,200 rpm for 3 minutes, the medium was aspirated and the cells re-suspended in PBS to a final concentration of 1 x 10^7 cells/ml. 1 x 10^7 ES cells were mixed with 15 µg of the targeting vector's DNA and immediately transferred into a 0.4 cm gap cuvette (Biorad, cat #: 165-2081). The electroporation was carried out using a Biorad "Gene Pulser" (Biorad, cat #: 165-2660) at 230 V, 500 µF (AB2.2 cells) or at 800 V, 25 µF (E14Tg2a cells). After electroporation, the cuvette was left for at least 5 minutes in the tissue culture (TC) hood before the ES cells were plated onto either 10 cm feeder plate (AB2.2 ES cells) or pre-gelatinised 10 cm non-feeder plate (E14Tg2a ES cells; gelatin was added to the flask/plates and left for at least 5 minutes before being aspirated; see **Table 2.1** for the appropriate gelatin quantity). Drug selection (G418 or puromycin depending on the targeting vector) was usually initiated 24 hours post-electroporation and, unless stated otherwise, continued for 10 days to allow the formation of single ES cell colonies. When drug selection was finished, the plate was used to pick single ES cell colonies.

2.2.1.3 PICKING ES CELL COLONIES

The medium was aspirated from the 10 cm plate and, after rinsing with PBS, 10 ml of PBS was added to the plate to cover the cells. Individual colonies were picked using a Pipetman® and added into a well of a 48-well round bottom plate, which was filled with 100 μl of trypsin. Then the plate was incubated in a TC incubator at 37°C for 4 minutes before adding 500 μl of fresh M15 medium to each well. Next, vigorous pipetting separated the ES colonies into single cells, and the ES cell suspension was then transferred to a replicate 48-well plate and incubated at 37°C with 5% CO_2.

2.2.1.4 PASSAGING ES CELLS

ES cells were ready for passaging when they reached approximately 80% confluence. At this time, the medium was aspirated and the flask/plate washed with PBS. 1x trypsin was added (according to **Table 2.1**) and the flask/plate incubated at 37°C for 4 minutes. M15 medium was then added to the flask/plate (according to **Table 2.1**) and the ES cell colonies were dispersed into single cells by pipetting the culture up and down. The ES cell culture suspension was then split into a 1:4 ratio and distributed between 2 feeder or 2 pre-gelatinised non-feeder flasks/plates for AB2.2 ES cells or 4 pre-gelatinised non-feeder flasks/plates for E14Tg2a ES cells (gelatin was added to the flask/plates and left for at least 5 minutes before being aspirated; see **Table 2.1** for the appropriate gelatin quantity). Two pre-gelatinised non-feeder flasks/plates were then used for extraction of genomic DNA (gDNA) from ES cells of both lines for subsequent analysis with Southern blotting (see sections), whilst the other 2 plates were used for expansion of both lines of ES cells. The flasks/plates were incubated in a TC incubator at 37°C with 5% CO_2.

Table 2.1. Volumes.

Flask/Plates	Gelatin	1x trypsin	Re-suspension medium final volume	Culture medium
T25 flask	5 ml	1 ml	10 ml	10 ml
T75 flask	10 ml	3 ml	15 ml	40 ml
T150 flask	15 ml	5 ml	25 ml	60 ml
10 cm plate	10 ml	3 ml	15 ml	40 ml
48-well plate	250 μl	100 μl	500 μl	500 μl

2.2.1.5 FREEZING ES CELLS

ES cells were ready for freezing when they reached about 80% confluence. ES cells were fed 3 hours before freezing, after which time the medium was aspirated, and the flask/plate washed once with PBS before trypsin was added (according to **Table 2.1**). The flask/plate was then incubated at 37°C for 4 minutes, before M15 medium was added (according to **Table 2.1**) to re-suspend the ES cells. The ES cell colonies were separated into single cells by gently pipetting the culture up and down, and the ES suspension then transferred into a sterile tube and centrifuged at 400 rpm for 5 minutes. Next, the supernatant was aspirated and ES cells were re-suspended in an appropriate volume of the freeze medium (according to **Table 2.1**) and immediately transferred into cryo-vials (Fisher Scientific, cat #: 375353) with a maximum of 1 ml per vial. The cryo-vials were then quickly transferred to an -80°C freezer and after 24 hours transferred to a liquid nitrogen tank for long-term storage.

2.2.1.6 THAWING ES CELLS

ES cells were removed from freezer/liquid nitrogen and thawed quickly (by rolling between the hands). The thawed ES cell suspension was transferred to a sterile tube containing less than 10 ml of warm medium, which was then centrifuged at 300 rpm for 5 minutes. The supernatant was aspirated and ES cells re-suspended in 10 ml of warm ES medium and plated onto T25 feeder flasks (AB2.2 ES cells) or to T25 pre-gelatinised non-feeder

flasks (E14Tg2a ES cells). The flask was incubated at 37°C with 5% CO_2. The ES cells were re-fed daily with fresh M15 medium and, once 80% confluency was reached, cells were passaged, frozen or used for other experiments.

2.2.1.7 ELECTROPORATION OF THE ES CELLS WITH THE pOG231 CRE-EXPRESSION VECTOR

20 µg of the pOG231 Cre-expression vector (O'Gorman 1997) was electroporated into 3.0 x 10^6 double-targeted AB2.2 ES cells using Biorad Gene Pulsar 2 at 230 V, 500 µF. The recombinants were first selected in HAT medium (M15 medium supplemented with 12 ml of 50x HAT) for 7 days and then recovered in HT medium (M15 medium supplemented with 12 ml of 50x HT) for 2 days. 48 clones were picked and expanded on a 48-well feeder plate. The plate was replicated, and sib-selection performed to determine the drug resistance of the clones. The correct recombinants should be G418−sensitive, puromycin−sensitive and HAT−resistant. The clones with this combination of drug resistance/sensitivity were expanded and confirmed by Southern blot analysis (as described in section 2.2.3.5).

2.2.1.8 PREPARING ES CELLS FOR MICROINJECTION

When ES cells reached approximately 80% confluence they were ready for micro-injections. The day prior to microinjection, the cells were passaged (as described above) to remove any poor quality, differentiated cells. On the day of microinjection, the medium was aspirated, the plate/flask washed once with PBS, and trypsin added (according to **Table 2.1**). The plate/flask was incubated at 37°C for 4 minutes before M15 medium was added (according to **Table 2.1**) to re-suspend the ES cells. The ES suspension was then transferred into a sterile tube and centrifuged at 400 rpm for 5 minutes. Next, the supernatant was aspirated and ES cells were re-suspended in 500 µl of Hepes/M15 medium. The suspension was mixed thoroughly to separate ES cells, transferred to a cryo-vial and immediately placed on ice to prevent the ES cells from adhering to each other.

2.2.2 GENERATION OF DELETION MICE

2.2.2.1 GENERATION OF THE *Df$^{Lipi\text{-}Usp25}$*/+ ES CELL LINE

The 5'*Hprt* MICER targeting vector MHPN69h23 (Adams 2004) and the 3'*Hprt* targeting vector (Pusp-3HPAg; constructed by recombineering to capture a 6963 bp fragment telomeric to *Usp25* from 129Sv BAC clone: bMQ134j07 (Adams 2005)) were used to generate a chromosomal deletion of a 1.6 Mb of mouse chromosome 16 syntenic to the human region 21q11.2–q21.1. Approximately 15 µg of MHPN69h23 plasmid was linearized with *Nhe*I and electroporated into E14Tg2a ES cells. Stable integrants were selected in M15 medium containing G418 (175 µg/ml) and positive clones (that had undergone homologous recombination with the target locus) were identified by Southern blotting using a probe amplified with the primers 5'-AGG CAA AAA CCA AGA CCT CA-3' and 5'-ATG GTG GCA ATG TTC TCA CA-3' on *Stu*I digested genomic ES cell DNA. The expected lengths of Southern restriction fragments were 15 and 17 kb for wildtype and targeted alleles respectively. Then approximately 15 µg of Pusp-3HPAg plasmid was linearized with *Swa*I and electroporated into MHPN69h23-targeted E14Tg2a ES cells and selected in M15 medium containing puromycin (3 µg/ml). Southern blotting was performed to identify positive recombinants using a probe amplified with the primers 5'-GTG CCC ACA TGG TTT TCT TT-3' and 5'-CAA CTC TCG CCT CAC ACA AA-3' on *Bam*HI digested genomic ES cell DNA. The expected lengths of Southern restriction fragments were 12 and 22.6 kb for wildtype and targeted alleles respectively. The double-targeted E14Tg2a ES cell clones were electroporated with the pOG231 Cre-expression vector (O'Gorman 1997) and selected in HAT medium (Ramirez-Solis 1995; Liu 1998). Subsequently, the HAT-resistant ES cell clones were selected with G418 and puromycin to identify the clones sensitive to both drugs, and so ES cell clones with both targeting vectors were inserted in *cis*. Finally, the presence of the desired deletion between *Lipi* and *Usp25*, generated by *cis*-recombination, was confirmed by both FISH (see section 2.2.2.3 below for details) and PCR using the primers

5’-AAG GGT GTT TAT TCC CCA TGG ACT AAT TAT G-3’ and 5’-CCT TCA TCA CAT CTC GAG CAA GAC GTT CAG-3’. The expected size of the PCR product was 1869 bp for the deletion allele. The deletion allele was designated $Df^{Lipi\text{-}Usp25}$ and ES cell clones carrying $Df^{Lipi\text{-}Usp25}$/+ were injected into albino C57BL/6J$^{c/c}$ blastocysts for germline transmission as described previously (Ramirez-Solis 1995).

2.2.2.2 GENERATION OF THE CONDITIONAL $Df^{4732471D19Rik\text{-}B4galt7}$/+ ES CELL LINE

The 5’*Hprt* MICER targeting vector MHPN55m07 (Adams 2004) and the 3’*Hprt* MICER targeting vector MHPP265c24 (Adams 2004) were used to generate a chromosomal deletion of a 1.1 Mb of mouse chromosome 13 syntenic to the human region 5q35.2–q35.3. Approximately 15 µg of MHPN55m07 plasmid was linearized with *Kpn*I and electroporated into AB2.2 ES cells. Stable integrants were selected in M15 medium containing G418 (175 µg/ml). Southern blotting was performed to identify positive recombinants using a probe amplified with the primers 5’-GTC TGT TGT TAA AAG CTA AAA CCT TAG A-3’ and 5’-TGA GCT ACA GTT TGG TTC TGG TGG ATA AAC-3’ on *Bst*EII-digested genomic ES cell DNA. The expected lengths of Southern restriction fragments were 8.5 and 24.5 kb for wildtype and targeted alleles respectively. Then approximately 15 µg of MHPP265c24 was linearized with *Nco*I and electroporated into MHPN69h23-targeted AB2.2 ES cells, which were subsequently selected in M15-containing puromycin (3 µg/ml). Southern blotting was performed to identify positive recombinants using a probe amplified using primers 5’-CAG TAA TAT AGT AGA AGC ATG GTC CAT-3’ and 5’-ATG ATA CTG AAC ACA GAC AAC AGA GGC TGC T-3’ on *Spe*I-digested genomic ES cell DNA. The expected lengths of Southern restriction fragments were 21 and 13 kb for wildtype and targeted alleles respectively. The double-targeted AB2.2 ES cell clones were electroporated with the pOG231 Cre-expression vector (O’Gorman 1997) and selected in HAT medium (Ramirez-Solis 1995; Liu 1998). Subsequently, the HAT-resistant ES cell clones were selected with G418 and puromycin to

identify the clones sensitive to both drugs, and so ES cell clones with both targeting vectors were inserted in *cis*. Finally, the presence of the desired deletion between *4732471D19Rik* and *B4galt7*, generated by *cis*-recombination, was confirmed by both FISH (see section 2.2.2.3 below for details) and PCR using the primers 5'-AAG GGT GTT TAT TCC CCA TGG ACT AAT TAT G-3' and 5'-CCT TCA TCA CAT CTC GAG CAA GAC GTT CAG-3'. The expected size of the PCR product was 1869 bp for the deletion allele. The deletion allele was designated $Df^{4732471D19Rik\text{-}B4galt7}$, and ES cell clones carrying the conditional deletion (i.e. not the actual cells that were electroporated with Cre) were injected into albino C57BL/6J$^{c/c}$ blastocysts to generate germline chimeras carrying the conditional deletion as described previously (Ramirez-Solis 1995). Subsequently, mice with the conditional deletion were bred with CAG-Cre mice to generate the $Df^{4732471D19Rik\text{-}B4galt7}$/+ mice.

2.2.2.3 FLUORESCENT IN SITU HYBRIDIZATION (FISH)

Chromosome spreads from ES cells were performed as described previously (Robertson 1987). Bacterial artificial chromosome (BAC) clones were used as probes for FISH (Baldini 1994). To detect ES cell clones carrying $Df^{Lipi\text{-}Usp25}$/+, BAC clone RP24-200E18 (located inside the deletion) and BAC clone RP23-246B23 (located outside the deletion) were used, while to detect ES cell clones carrying $Df^{4732471D19Rik\text{-}B4galt7}$/+, BAC clone RP23-99C7 (located inside the deletion) and BAC clone RP24-204D5 (located outside the deletion) were used. BAC clone RP24-200E18 and BAC clone RP24-204D5 were labelled with biotin and detected with three layers of antibodies: 1) Texas red avidin DCS (Vector Laboratories, cat. #: A-2016), 2) biotinylated anti-avidin D made in goat (Vector Laboratories, cat. #: BA-0300) and 3) Texas red avidin D (Vector Laboratories, cat. #: A-2006). In contrast, BAC clone RP23-246B23 and BAC clone RP23-99C7 were labelled with digoxigenin and detected with FITC conjugated mouse anti-dig monoclonal antibody and further amplified with rabbit or rabbit anti-mouse IgG (4 µg/ml Texas red avidin DCS; Vector Laboratories, cat. #: A-2016, 4 µg/ml biotinylated anti-avidin D; Vector Laboratories, cat. #: BA-0300 plus 1 µg/ml mouse anti-digoxigenin; LifeSpan BioSciences, cat. #: LS-C64857) or

1:500 – 1000 dilution of mouse anti-digoxin (Sigma-Aldrich, cat. #: D8156), 4 µg/ml Texas Red Avidin DCS (Vector Laboratories, cat. #: A-2016) plus 10 µg/ml goat anti-mouse IgG (whole molecule) – FITC antibody (Sigma-Aldrich, cat. #: F0257) or 7.5 µg/ml rabbit anti-mouse IgG (whole molecule) – FITC antibody (Sigma-Aldrich, cat. #: F7506)).

2.2.2.4 USE OF ANIMALS

Mice were treated in accordance with local ethical committee guidelines and the UK Animals (Scientific Procedures) Act 1986, and all procedures were carried out in accordance with Home Office guidelines (United Kingdom).

2.2.3 DNA METHODS

2.2.3.1 PURIFICATION OF BAC DNA

Glycerol stocks of BAC clones were inoculated in 1 – 2 ml of lysogeny broth (LB) with kanamycin (30 µg/µl) and left in a shaking incubator at 37°C overnight. The next day, 1 ml of the overnight culture was inoculated in 50 – 100 ml of LB with kanamycin (30 µg/µl) and left in a shaking incubator at 37°C overnight. The BAC DNA was extracted using Plasmid Plus Maxi Kit (Qiagen, cat. #: 12963) according to the manufacture's instructions. The overnight culture was centrifuged at 16,000 rpm for 5 minutes and the pellet was then re-suspended in 8 ml of Buffer P1. Next, 8 ml of Buffer P2 was added to the mixture, gently mixed by inverting until the lysate appeared viscous, and incubated at room temperature for 3 minutes. The QIAfilter Cartridge was placed into a new tube, and 8 ml of Buffer S3 was added to the lysate and mixed by inverting 4 – 6 times. The lysate was then transferred to the QIAfilter Cartridge and incubated at room temperature for 10 minutes. During incubation, QIAGEN Plasmid Plus spin columns were placed into the QIAvac 24 Plus and Tube Extenders were inserted into each column. Next, the plunger was gently inserted into the QIAfilter Cartridge and the cell lysate

was filtered into the tube. 5 ml Buffer BB was added to the cleared lysate and mixed by inverting 4 – 6 times. The lysate was transferred to a QIAGEN Plasmid Plus spin column on the QIAvac 24 Plus. Approximately a 300 mbar vacuum was applied until the liquid had been drawn through all columns. The DNA was washed, 0.7 ml Buffer ETR was added and a vacuum was applied until the liquid had been drawn through all the columns. 0.7 ml Buffer PE was added and a vacuum was applied until the liquid had been drawn through all columns. The column was centrifuged at 10,000 rpm for 1 minute in a tabletop microcentrifuge to completely remove residual wash buffer. The QIAGEN Plasmid Plus spin column was placed into a clean 2 ml tube. The DNA was eluted by adding 400 µl Buffer EB or H_2O to the centre of the QIAGEN Plasmid Plus spin column followed by centrifuging for 1 minute.

2.2.3.2 EXTRACTION OF GENOMIC DNA FROM ES CELLS

ES cells were incubated in 150 µl of lysis buffer [50 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.4), 50 mM EDTA (pH 8), 0.5% Sodium Dodecyl Sulfate (SDS), and 1 mg/ml proteinase K (added fresh)] at 55°C – 65°C overnight in a humidified chamber. The next morning the ES cell suspension was centrifuged at 4,000 rpm for 3 minutes. Then the supernatant was added to a fresh tube containing 250 µl of 100% ethanol and mixed by inversion to precipitate until gDNA was seen. To pellet gDNA, samples were centrifuged at 13,000 rpm for 1 minute. Then the supernatant was discarded, 1 ml of 70% ethanol was added to the gDNA pellet, mixed by inverting the tube several times, and centrifuged at 13,000 rpm for 1 minute. Finally, the supernatant was discarded, and the gDNA pellet was air-dried before being resuspended in an appropriate volume of 10 mM Tris-HCl (pH 7.4).

2.2.3.3 EXTRACTION OF GENOMIC DNA FROM EAR OR TAIL BIOPSIES

Ear or tail biopsies were incubated in 150 µl (ear) or 500 µl (tail) of lysis buffer [50 mM Tris (pH 7.4), 50 mM EDTA (pH 8), 0.5% SDS, and 1 mg/ml proteinase K (added fresh)] at 55°C – 65°C overnight. The next morning samples were centrifuged at 13,000 rpm for 3 minutes to pellet down

fur and undigested tissue. Then the supernatant was added to a fresh tube containing 250 µl (ear) or 900 µl (tail) of 100% ethanol and mixed by inversion to precipitate the gDNA. To pellet the gDNA, samples were centrifuged at 13,000 rpm for 1 minute. Then the supernatant was discarded, 1 ml of 70% ethanol was added to the gDNA pellet, mixed by inverting the tube several times, and centrifuged at 13,000 rpm for 1 minute. Finally, the supernatant was discarded, and the gDNA pellet was air-dried before being resuspended in an appropriate volume of 10 mM Tris-HCl (pH 7.4).

2.2.3.4 POLYMERASE CHAIN REACTION (PCR)

2.2.3.4.1 DESIGN AND SYNTHESIS OF PRIMERS FOR PCR

Oligonucleotides were designed using the Primer3 design package hosted by the Whitehead Institute for Biomedical Research at http://frodo.wi.mit.edu/primer3/. Primers were ordered from Sigma-Aldrich. Primers were diluted with H_2O to a stock concentration of 100 µM. Working dilutions were then made to a concentration of 10 µM (see **Table 2.2** for primer sequences).

Table 2.2. PCR primer sequences.

Name	5' to 3'	Length (bp)
Del_*Hprt*_F	AAGGGTGTTTATTCCCCATGGACTAATTATG	1869
Del_*Hprt*_R	CCTTCATCACATCTCGAGCAAGACGTTCAG	
Del_vector_F	ACACCAGAACCCTAGCATGG	659
Del_vector_R	GCCTACATACCTCGCTCTGC	
Del_F	CTAGTACAGTCGGTAAGAACAAAATAGTGTCTATCAATAGTGGACTGG	898
Del_R	GGTGTTTATTCCCCATGGACTAATTATGGACAGG	
Mosaic_F	CTGTACACATTTCTTCTCAAGCACTGGCTATGCATGTATAC	356
Mosaic_R	CACCGCTGAATATGCATAAGGCAGGCAAGATGGCGCGTCC	
Cre_F	GTCGATGCAACGAGTGATGAG	881
Cre_R	ATCTCCGGTATTGAAACTCCAGC	
*DfLipi-Usp25*_wt_F	CCTGTGGCCAATTCAAAAGT	526
*DfLipi-Usp25*_wt_R	TTCAGCTGGCCTTTTTCACT	
*DfRik-B4galt7*_wt_F	CTTCCTGCCTCAACCTCTTG	698
*DfRik-B4galt7*_wt_R	TTGAAGCTCACAGTGCCTTG	
Bloom_F1	TCATTTTGGCAGTCCACCTC	300
Bloom_R1	GTCGCTCTAATCCTTTCCATTC	
Bloom_F2	CTCACCAGATAGCAAGCAG	2700
Bloom_R3	TTAAGACCAGGGCTAGACAG	
MICEpuro	CTAGTACAGTCGGTAAGAACAAAATAGTGTCTATCAATAGTGGACTGG	330
MICERcmn	CTGTACACATTTCTTCTCAAGCACTGGCTATGCATGTATAC	
MICERneo	CACCGCTGAATATGCATAAGGCAGGCAAGATGGCGCGTCC	356
MICERcmn	CTGTACACATTTCTTCTCAAGCACTGGCTATGCATGTATAC	
MHPN55m07_F	GTCTGTTGTTAAAAGCTAAAACCTTAGA	506
MHPN55m07_R	TGAGCTACAGTTTGGTTCTGGTGGATAAAC	
MHPP265c24_F	CAGTAATATAGTAGAAGCATGGTCCAT	450
MHPP265c24_R	ATGATACTGAACACAGACAACAGAGGCTGCT	
MHPN69h23_F	AGGCAAAAACCAAGACCTCA	455
MHPN69h23_R	ATGGTGGCAATGTTCTCACA	
3'fragment_F	GTGCCCACATGGTTTTCTTT	429
3'fragment_R	CAACTCTCGCCTCACACAAA	

2.2.3.4.2 GENERAL PCR PROTOCOLS

Two different PCR systems were used to carry out PCR reactions. The first system, ABgene Thermo-Start™ *Taq* DNA Polymerase (Thermo Scientific, cat. #: AB0908/A), was used for the majority of PCR reactions. The second system, *TaKaRa LA Taq*™ (Takara Biotechnology, cat. #: RR002A),

was used for reactions where a larger fragment was to be amplified.

The reaction mixture for the PCR reactions carried out using ABgene Thermo-Start™ *Taq* DNA Polymerase was prepared by combining 2.5 µl 10x ABgene Thermo-Start PCR Buffer, 1.5 µl $MgCl_2$ (25 mM), 0.5 µl dNTP mixture (20 mM), 0.125 µl ABgene Thermo-Start *Taq* DNA Polymerase (5 U/µl), 0.5 µl of each forward and reverse primer (10 µM), 5 – 100 ng DNA template, and an appropriate volume of double-distilled H_2O (ddH_2O) to bring the reaction to a final volume of 25 µl. The cycling conditions were: 95°C for 15 minutes (initial denaturation), 30 – 40 cycles of denaturation (95°C for 30 seconds) then annealing (55 – 65°C for 30 – 60 seconds) then extension (72°C for 30 – 90 seconds), followed by 72°C for 5 – 10 minutes (final extension). The annealing temperature depended on the primer pair used, while the extension time was 1 minute for every 1 kb of target fragment.

The reaction mixture for the PCR reactions carried out using *TaKaRa LA Taq*™ were prepared by combining 2.1 µl 10x TaKaRa LA™ Buffer II (Mg^{2+} free), 2.1 µl $MgCl_2$ (25 mM), 3.36 µl dNTP mixture (2.5 mM of each dNTP), 0.17 µl *TaKaRa LA Taq*™ DNA Polymerase (5 U/µl), 0.08 µl each of forward and reverse primer (10 µM), 5 – 100 ng DNA template, and appropriate volume of ddH_2O to bring a reaction to a final volume of 20 µl. The cycling conditions were: 94°C for 5 minutes (initial denaturation), 35 cycles of denaturation (94°C for 15 seconds) then annealing (56°C for 30 seconds) then extension (68°C for 2:30 minutes), followed by 68°C for 7 minutes (final extension).

The products of PCR reactions were mixed with 5x loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF in 30% glycerol (made up in ddH_2O)), and loaded onto ethidium bromide-containing 1 – 2% agarose gels (UltraPure™ Agarose (Invitrogen, cat. #: 16500-500) dissolved in 1x Tris-acetate-EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA)). The PCR bands were then visualised by placing the gel on a UV transilluminator.

2.2.3.5 SOUTHERN BLOTTING

2.2.3.5.1 Digestion of the DNA samples

5 – 10 µg of genomic DNA (in a total volume of no more than 40 µl) was digested overnight with 2 µl of an appropriate restriction enzyme, 5 µl 10x restriction buffer and sufficient ddH_2O to make a 50 µl final reaction volume. The digestion temperature depended on the requirements of the individual enzyme.

2.2.3.5.2 Electrophoresis and transfer of the DNA

The digested fragments, as well as one lane of molecular weight marker DNA (*Lambda DNA/HindIII markers*; Promega, cat. #: G1711), were separated by electrophoresis on an ethidium bromide-containing 0.8% agarose gel run at 60 – 80 V (depending on the width, length and thickness of the gel). Upon completion of electrophoresis, a fluorescent ruler was placed alongside the gel on the UV transilluminator, and the ruler and gel were photographed (to allow estimation of the size of the bands if the markers did not show up on the blot after hybridization). Next, the gel was washed twice for 10 minutes in depurination buffer (40 ml of concentrated HCl in 2 l of Mili-Q H_2O) and then once for 15 minutes in neutralization buffer (0.4 M NaOH, 1 M NaCl). The transfer of DNA samples from the gel to the membrane was achieved by capillary action. A strip of 3 MM Whatman paper was placed in the gel-running apparatus (the strip was slightly wider in width than the gel across the gel-running apparatus, allowing the ends of the paper to reach over the edge of the platform into a reservoir containing the neutralization buffer). Next, the gel was inverted (turned face-down) and placed on top of the Whatman paper on the gel box. On top of the gel was placed a sheet of positively charged nylon membrane (Amersham Hybond-XL; GE Healthcare Life Sciences, cat. #: RPN203S) that had been soaked in Mili-Q H_2O followed by neutralization buffer. Two pieces of Whatman paper (pre-soaked in Mili-Q H_2O) were placed on top of the membrane. Finally, a 10 – 15 cm layer of absorbent paper towel was placed on top of the Whatman paper. Following the overnight transfer, the transfer apparatus was disassembled and the membrane washed in wash buffer

(0.5 M Tris HCl, 1 M NaCl in Mili-Q H_2O) for 15 minutes. Then the membrane was placed inbetween some filter paper and baked at 80°C for 2 hours.

2.2.3.5.3 Hybridization, preparation of the probe and washing the blot

The membrane was pre-hybridized for 2 hours in Amersham Rapid-hyb™ Buffer (GE Healthcare Life Sciences, cat. #: RPN1636) at 65ºC in a rotating hybridization oven (about 10 – 15 ml of buffer was used per blot depending on its size). The probe was labelled using the Prime-It® II Random Primer Labeling Kit (Agilent Technologies, cat. #: 300385), according to the manufacturer's instructions. Briefly, 50 ng of probe was added to a Mili-Q H_2O to make 25 µl, and then 10 µl of Random 9-mer primers were added. The mixture was heated at 100ºC for 5 minutes and then placed on ice for 1 minute. Next, 10 µl of buffer, 1 µl of Klenow polymerase and 5 µl of αP^{32}–dCTP (=50 µCi) was added to the mixture, which was subsequently incubated at 37ºC for at least 15 minutes. Then, the probe was cleaned up through the Illustra ProbeQuant™ G-50 Micro column (GE Healthcare Life Sciences, cat. #: 28-9034-08). To do this, the bottom of the column was snapped off and the column placed in an eppendorf tube before being centrifuged at 4,000 rpm for 2 minutes. The column was then put in a new eppendorf tube and the probe was added to the gel resin that had pelleted in the column. After re-centrifugation, the column was discarded and the purified probe (i.e. the eluant) was heated at 100ºC for 5 minutes. The tube was then placed on ice for 1 minute, before being added to the pre-hybridized membrane. After 5 hours of hybridization at 65ºC, the blot was washed in the rotating tube with a low stringency wash (LSW; 2x SSC, 0.1% SDS) for 15 minutes, followed by a high stringency wash (HSW; 0.5x SSC, 0.1% SDS) for another 15 minutes. Then the blot was removed from the tube, wrapped in cling film, placed into a hybridization film cassette, where it was exposed to an X-ray film (Fisher Scientific, cat. #: P10M000274A) overnight at -80ºC. The film was then developed the next morning using the Compact X4 film processor (Xograph), according to the manufacturer's instructions.

2.2.4 RNA METHODS

2.2.4.1 TOTAL RNA EXTRACTION FROM TISSUES

Total RNA was isolated from mouse tissues using TRIzol (Invitrogen, cat. #: 15596-018) according to the manufacturer's instructions. Snap frozen tissues were transferred into RNase-free eppendorf tubes, where they were then homogenized in 1 ml of TRIzol (Invitrogen, cat. #: 15596-018). The homogenate was then centrifuged at 12,000 rpm at 4°C for 10 minutes and the supernatant transferred to a fresh RNase-free eppendorf tube and 200 µl of chloroform. The tube was vigorously shaken for 15 seconds, incubated at room temperature for 2 minutes and centrifuged at 12,000 rpm at 4°C for 10 minutes. The aqueous phase was removed and transferred to a fresh RNase-free eppendorf tube, and the RNA precipitated by adding 500 µl of isopropyl alcohol. The sample was incubated at room temperature for 10 minutes and then centrifuged at 12,000 rpm at 4°C for 10 minutes. The RNA pellet was washed once with 1 ml of 75% ethanol and centrifuged at 7,500 rpm at 4°C for 5 minutes. The air-dried RNA was re-suspended in an appropriate volume of RNase-free H_2O.

2.2.4.2 DNase TREATMENT OF RNA

RNA was DNase treated using TURBO^{TM} DNase kit (Ambion, cat. #: AM2238) according to the manufacturer's instructions. 12 µg of total RNA in a volume of 21.5 µl of RNase-free H_2O was mixed with 2.5 µl of 10x TURBO DNase buffer and 1 µl of TURBO DNase. The mixture was incubated at 37°C for 30 minutes, flicked and then incubated at 37°C for an additional 30 minutes. 6 µl of inactivation reagent was added and the mixture was vortexed before being incubated at room temperature for 5 minutes (while incubating, the content of the tube was flicked a few times). The tube was centrifuged at 12,000 rpm for 90 seconds. The supernatant was carefully transferred into a new tube and 50 µl of 100% ethanol was added. After inverting the tube several times, it was placed in a freezer for 10 minutes, before being centrifuged at 13,000 rpm at 4°C for 10 minutes. The pellet was air-dried for 5 minutes and then resuspended in 15 µl of RNase-free H_2O.

2.2.4.3 EXPRESSION ARRAY ANALYSIS

DNase-treated RNA was amplified using the Illumina® TotalPrep™-96 RNA Amplification Kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Expression profiling of the RNA was performed using a MouseWG-6 v2.0 expression beadchip kit (Illumina, Essex UK), according to the manufacturer's instructions. Microarray data was imported in the R programming language for processing. We used the lumi package [http://www.bioconductor.org/] to perform quality control on array data (samples were removed if there were concerns over their quality). These data were then quantile-normalised (Yang 2001) as implemented in the lumi package [http://www.bioconductor.org/packages/ 2.0/bioc/html/lumi.html] and limma package (Smyth 2004). Data were p-value adjusted to yield a sorted list of differentially expression genes (Benjamini 2001).

2.2.4.4 FIRST STRAND COMPLEMENTARY DNA (cDNA) SYNTHESIS

1 µg of total RNA was made up to 21 µl with RNase-free H_2O in an RNase-free eppendorf tube. Next, 20 µl of the mixture was added to Sprint^{TM} RT Complete Product tube (Clontech, cat. #: 639525), mixed by pipetting, and incubated at 42°C for 1 hour. The tube was then incubated at 70°C for 10 minutes, to terminate the reaction.

2.2.4.5 REAL-TIME QUANTITATIVE PCR (qRT-PCR)

The quantitative PCR reactions were performed with SYBR® Green PCR Master Mix (Applied Biosystems, cat. #: 4309155) on the ABI 7900HT sequence detection system, according to the manufacturer's instructions. 12.5 µl of SYBR® Green PCR Master Mix was mixed with 1 µl of forward primer, 1 µl of reverse primer (see **Table 2.3** for qRT-PCR primers sequences), 8.5 µl of ddH_2O and 2 µl of cDNA. The cycle conditions were: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and then 60°C for 1 minute, followed by 95°C for 15 seconds. The final quantitation was determined relative to the average CT of the house-keeping genes *Eif1a*, *Hprt1* and *Gapdh* (Livak 2001). Data were statistically analysed using the two-tailed Student's *t*-test.

Table 2.3. qRT-PCR primer sequences.

Gene	Forward: 5' to 3'	Reverse: 5' to 3'
Eif1a	AAAAACAGGCGCAGAGGTAAA	TCCTCACACCGTCAAAGCAC
Hprt1	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Tmem45a	TGGGCTTTTGGTGGACTATGA	CCAGCTATACCAGTGAGAGACA
Plnxd1	TCGCTGCCAATCCCTAATAAGA	TGACCTGGTTTGGAACTGTTG
Elovl1	TCCAAAGCTACCCTCTGATGG	AGGGAGAGTATCACCAGTGAGA
Man2b2	AAAGCATGAGAGCCTATGCAG	GCCTCCGAGAACAAACTCCA
Atp5g3	TCTGCATCAGTGTTATCTCGGC	CACCAGAACCAGCAACTCCTA
Mrap	ACATAGACCTCATTCCTGTGGA	TGTGTTCCGACTTACGCTTGT
Rbm38	TGCTCCCCGAGTGTGTTTC	GTACTTTCTGAGCGATGCGTC
Tnxb	TCCGTGTAGACTCAGCAAAGG	CCCCACGATAAGAGACAGCG
Gtf2ird1	TTCGTCCTCTAACCCAGAGTC	ACAGAATTAGGGTGAAGTTCGGA
Thbs3	ATGGAGAAGCCGGAACTTTGG	AGTGAGTAAAGCTGTCCGAATCT
Lrpap1	CACAACCTCAACGTCATCCTG	AGCACATTGTACTCCTGGATCTT
Trpc4ap	ACATGGCTCGACAATGCGTT	TGCTTAGAGAAGGGAACACAGT
Samsn1	CCAAGTCCCTATGACACCGAC	CCTGGATAGTCTGGTGGTTCT
Lmna	ACCCCGCTGAGTACAACCT	TTCGAGTGACTGTGACACTGG
Bmp1	TTGTACGCGAGAACATACAGC	CTGAGTCGGGTCCTTTGGC

2.2.5 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

2.2.5.1 COLLECTION AND EMBEDDING OF TISSUES/EMBRYOS

Mouse tissues and embryos (at E10.5, E11.5, E12.5 and E14.5) for histopathological analysis were collected into 10% neutral buffered formalin, embedded in paraffin and 5 μm sections placed on glass slides. The only exception was for bones, which were decalcified in 10% EDTA for 7 days prior to embedding in paraffin.

2.2.5.2 HEMATOXYLIN AND EOSIN (H&E) STAINING

The sections were first de-waxed using xylene washes (1x for 3 minutes and 1x for 2 minutes), subsequently re-hydrated in a decreasing series of industrial methylated spirit (IMS) (Leica, cat. #: 03655EG) dilutions

(2x in 100% IMS for 1 minute and 1x in 70% IMS for 1 minute) and rinsed in ddH_2O for 1 minute before staining with hematoxylin and eosin. The staining protocol was as follows: Harris's Hematoxylin solution (Raymond A Lamb, cat. #: 10874) for 3 minutes, tap H_2O for 1 minute, 1% acid alcohol for 20 seconds, ddH_2O for 30 seconds, tap H_2O for 3 minutes, 1% eosin (Raymond A Lamb, cat. #: 10814) for 3 minutes, and H_2O for 10 seconds. Then, the slides were dehydrated in an increasing series of IMS dilutions (1x in 70% IMS for 2 minutes, 2x in 100% IMS for 1 minute), cleared using xylene washes (2x for 90 seconds), and finally covered using the Thermo Scientific Shandon Synthetic Mountant (Fisher HealthCare, cat #: 6769007).

2.2.5.3 VON KOSSA STAINING

The sections were first de-waxed using xylene washes (1x for 3 minutes and 1x for 2 minutes), subsequently re-hydrated in a decreasing series of industrial methylated spirit (IMS) (Leica, cat. #: 03655EG) dilutions (2x in 100% IMS for 1 minute and 1x in 70% IMS for 1 minute) and rinsed in ddH_2O for 1 minute before staining with the von Kossa kit (Diagnostic BioSystems, cat. #: KT028) according to the manufacturer's instructions. Briefly, the slides were incubated in 5% silver nitrate solution for 24 hours in complete darkness, rinsed in ddH_2O, stained with 5% sodium thiosulfate solution for 3 minutes, rinsed in running tap H_2O, and nuclear fast red stained for 5 minutes. Then, the slides were dehydrated in an increasing series of IMS dilutions (1x in 70% IMS for 2 minutes, 2x in 100% IMS for 1 minute), cleared using xylene washes (2x for 90 seconds), and finally cover-slipped using the Thermo Scientific Shandon Synthetic Mountant (Fisher HealthCare, cat #: 6769007).

2.2.5.4 IMMUNOHISTOCHEMISTRY

The sections were first de-waxed using xylene washes (1x for 5 minutes and 1x for 4 minutes), subsequently rehydrated in a decreasing series of industrial methylated spirit (IMS) (Leica, cat. #: 03655EG) dilutions (1x in 100% IMS for 3 minutes, 1x in 100% IMS for 1 minute and 1x in 70% IMS for 1 minute), rinsed in ddH_2O for 1 minute, and finally moved to tap H_2O. Then, the sections were boiled in a pre-heated 10 mM citric acid buffer

(1.92 g of citric acid anhydrous (Sigma-Aldrich, cat. #: 251275) in 1 l ddH_2O, pH6 with NaOH (Sigma-Aldrich, cat. #: 221465)) in a microwave set up at 750 W for 10 – 15 minutes. The slides were gradually cooled in running tap H_2O for 10 minutes. Next, the sections were put in a 3% hydrogen peroxide (Sigma-Aldrich, cat. #: H1009-500ML) solution for about 20 minutes. The slides were put into Tris Buffered Saline with Tween 20 (TBST) solution (50 ml of 1 M Tris (pH 7.4), 30 ml of 5 M NaCl (Analar, cat. #: 10241), 500 μl of Tween 20 (Sigma-Aldrich, cat. #: 7949) diluted in 1 l ddH_2O) for 5 minutes. Wax barriers were drawn around the tissue sections on each slide using a pap pen (Vector laboratories, cat. #: H-4000). Approximately 100 μl of Blocking solution (15 μl of goat serum (VECTASTAIN®EliteABC Kit (Rabbit IgG); Vector Laboratories, cat. #: PK6101) in 1 ml of TSBT) was then applied to each section, which was then incubated in a humidified chamber at room temperature for 30 minutes. Then the blocking solution was tipped away, and each section incubated with approximately 100 μl mixture of the primary antibody in TBST (cleaved caspase-3 (Asp175) Antibody (Cell Signaling, cat #: 9661S) was used at 1:50 dilution in TBST) in a humidified chamber at room temperature for 1 hour. Following the incubation with the primary antibody, the sections were washed in TBST for 5 minutes and incubated with approximately 100 μl mixture of the secondary antibody in TBST (5 μl of secondary antibody (VECTASTAIN®EliteABC Kit (Rabbit IgG); Vector Laboratories, cat. #: PK6101) diluted in 1 ml of TBST) and incubated in the humidified chamber at room temperature for 30 minutes. The sections were then washed with TSBT for 5 minutes and incubated with approximately 100 μl of a mixture consisting of 10 μl of vector A (VECTASTAIN®EliteABC Kit (Rabbit IgG); Vector Laboratories, cat. #: PK6101) and 10 μl of vector B (VECTASTAIN®EliteABC Kit (Rabbit IgG); Vector Laboratories, cat. #: PK61010) diluted in 1 ml of TBST in a humidified chamber at room temperature for 45 minutes. Next, the sections were washed with TSBT for 15 minutes. To develop a colour, each section was incubated with approximately 100 μl mixture consisting of 5 ml of ddH_2O, 2 drops of Buffer Stock Solution (Vector Laboratories, cat #: SK-4100), 4 drops of DAB Stock Solution (Vector Laboratories, cat #: SK-4100) and 2 drops of Hydrogen

Peroxide Solution (Vector Laboratories, cat #: SK-4100). The sections were then washed with TSBT for 5 minutes, stained with hematoxylin solution for 2 minutes and placed in tap H_2O. Then, the slides were dehydrated in a decreasing series of IMS dilutions (1x in 100% IMS for 2 minutes, 1x in 100% IMS for 2 minutes and 1x in 70% IMS for 1 minute), cleared using xylene washes (2x for 90 seconds), and finally cover-slipped using the Thermo Scientific Shandon Synthetic Mountant (Fisher HealthCare, cat #: 6769007).

2.2.5.5 FISH ANALYSIS ON TISSUE SECTIONS MOUNTED ON GLASS SLIDES

The slides were first de-waxed using xylene washes (3x for 5 minutes), subsequently re-hydrated in 100% industrial methylated spirit (IMS) (Leica, cat. #: 03655EG) (2x for 5 minutes), and air-dried. Next, the sections were subjected to pre-treatment for digestion, which consisted of incubation in 0.2 M HCl (100 ml of 1 M HCl to 400 ml of ddH_2O) for 26 minutes, washing in ddH_2O for 3 minutes, incubation in 10 mM citric acid (0.768 g of citric acid to 400 ml of ddH_2O; pH 6.0 using 10% sodium hydroxide) at 82°C for 3 hours, and washing in ddH_2O for 3 minutes. Subsequently, the slides were blot dried and subjected to proteinase K digestion. Namely, 500 μl of 0.5 μg/ml proteinase K solution was added to the pre-heated proteinase K buffer solution (50 ml of 2x saline-sodium citrate (SSC); pH 7.0) at 39°C and subsequently the mixture was applied to the slides. The slides were digested for 22 minutes, washed in ddH_2O for 3 minutes, dehydrated through 70%, 90% and 100% IMS each for 1 minute, and air-dried. Next, 6 – 10 μl of FISH probe was added per slide and then the slide was cover-slipped and sealed with rubber cement (Elmer's, cat. #: 231). Slides were put in the Slide Moat™ (Boekel Scientific, cat. #: 240000) and double-stranded DNA (dsDNA) was denaturated at 75°C for 5 minutes and then hybridized at 37°C overnight (14 –20 hours). The next day, the rubber cement was removed and the slides were placed into 2x SSC to remove coverslips. Then, the slides were blot-dried, washed in post hybridization buffer (100 ml of 20x SSC, 847 ml ddH_20 and 3 ml of Tergitol-type nonyl phenoxypolyethoxylethanol-40

(NP-40); pH 7.0) at 72°C for 4 minutes, rinsed in ddH_2O for 1 minute, and dried at 45°C using the Slide Moat™. Finally, 10 μl of 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain was added to the slide and then the slide was cover-slipped and sealed with nail varnish (Miss Sporty, cat. #: 105901). The slides were stored at 4°C and scored within 7 days.

2.2.6 PHENOTYPIC PROCEDURES

14 heterozygous (monosomic) *Df*$^{Lipi\text{-}Usp25}$ mice and 14 controls (wildtype littermates) fed on a high-fat diet (HFD) from 4 weeks of age were subjected to a robust phenotypic screening program in order to thoroughly determine whether clinical features diagnosed in patients with Monosomy 21 syndrome could be observed in monosomic *Df*$^{Lipi\text{-}Usp25}$ mice. See **Table 2.4** for a brief summary of the phenotypic tests. Members of the Wellcome Trust Sanger Institute Mouse Genetics Project performed these tests, while I generated the mouse cohort, genotyped all mice prior to testing and was present in an observational capacity when all the tests were conducted. Also, I statistically analysed all the results that were generated from the tests and interpreted the data.

Table 2.4. Short summary of the phenotypic tests performed on 14 monosomic *Df*$^{Lipi\text{-}Usp25}$ mice and 14 wildtype littermates fed on a high-fat diet.

Time	Name of the test	Purpose of the test
Weekly; from 4-week old	Weighting mice	To monitor the body weight
4-week old	Hair dysmorphology	To check for the coat formation and development
6-week old	Hair follicle cycling	To investigate the hair follicle cycling
9-week old	Open field	To measure the locomotion, habituation and fear/anxiety responses to a novel environment
9-week old	SHIRPA	To assess for the presence of gross motor and neurological abnormalities
9-week old	Grip strength	To assess the neuromascular function and muscular strength of the fore and all paws
10-week old	Dysmorphology	To check for the presence of any gross dysmorphological abnormalities
10-week old	Hot plate	To assess for the thermal pain perception
12-week old	Indirect calorimetry	To investigate mouse metabolism, circadian pattern and behaviour (mouse activity and to some extent its exploratory behaviour)
13-week old	Glucose tolerance	To obtain fasted, basal blood glucose concentrations To investigate glucose tolerance and clearance
14-week old	Auditory brainstem response	To assess the hearing sensitivity across a broad range of frequencies
14-week old	Dual energy X-ray absorptiometry (DEXA)	To obtain the body composition and bone mineral data
14-week old	X-ray imaging	To obtain high-resolution X-ray images of the mouse skeleton
15-week old	Core temperature stress	To obtain the basal body temperature To assess the stress-induced hyperthermia
15-week old	Eye morphology screen	To detect gross morphological abnormalities in the eye morphology
16-week old	Heart weights	To assess the weight of the mouse's heart
16-week old	Haematology panel	To analyse the variable whole blood parameters
16-week old	Plasma chemistry panel	To analyse the variable clinical chemistry parameters

Note: DEXA was also performed on 8- or 25-week old mice fed on a high-fat diet, and on 8-, 25-week old or on 1-year old mice fed on a normal-fat diet. X-ray was also performed on 1-year old mice fed on a normal-fat diet.

2.2.6.1 ANAESTHESIA WITH KETAMINE/XYLAZINE AND ANTISEDAN

When the mouse needed to be placed under general anaesthesia, it was weighed and 100 μl ketamine/xylazine and antisedan (Sigma-Aldrich) was injected intraperitoneally per 10 g of body weight. After administering anaesthesia, the mouse was put into a clean cage placed on top of a heat mat, and anaesthesia confirmed by checking for a righting reflex. After the test procedure was finished, but at least 15 minutes after anaesthesia was administered, antisedan was administered to reverse the anaesthetic state. The mouse was returned to the cage and placed on its side to help in breathing. The cage was placed within the Techniplast heated IVC recovery rack and mice in the cage were checked every 30 minutes. Mice were usually fully recovered within 1 – 2 hours after the injection of antisedan.

2.2.6.2 DIET TYPES

2.2.6.2.1 Normal-fat diet

Unless otherwise indicated, mice were fed on a standard laboratory chow (a normal-fat diet; NFD) (Autoclavable Mouse Breeder Diet 5021, a version of Mouse Diet 9F 5020; Lab Diet, cat. #: 5021) containing not less than 9% crude fat, 20% crude protein and 63% carbohydrate.

2.2.6.2.2 High-fat diet

A small fraction of mice were fed on a high-fat diet (HFD) (Western RD; Special Diets Services, cat. #: 829100) containing 21.4% crude fat, 17.5% crude protein and 50% carbohydrate from 4 weeks of age.

2.2.6.3 WEIGHING MICE

Mice were weighed weekly from 4 weeks of age to obtain and monitor their body weight over 12 subsequent weeks. Each mouse was placed in a container on the scales. The scale then counted down from 5 seconds and produced an average weight read-out on the display. The container was cleaned with 70% ethanol after weighing all mice from the same cage.

2.2.6.4 HAIR DYSMORPHOLOGY

This analysis was performed to check for coat formation and development in 4-week old mice. First, the back of the mouse was examined for the presence of guard hair. Next, the dorsal and ventral coat was checked for the presence of abnormalities, such as long, short, rough, sparse, coarse, fine hair and/or visible skin. Subsequently, the area behind the mouse's ears was examined for the presence or absence of hair. The tail was checked for the presence or absence of hair along its length, as well as the tip. Finally, the whiskers were checked for the presence of abnormalities, such as short, long, curly, disorientated, and/or absent hairs.

2.2.6.5 HAIR FOLLICLE CYCLING

This test was performed to investigate hair follicle cycling. This test is dependent on the mouse age and therefore all mice were tested when they were 43 days old. This time point was chosen on the basis of previous internal data, which suggested that at 43 days of age, a mouse is the most likely to leave the anagen phase and enter the catagen phase, a phase which can be recognised by a skin colour change from black to grey-pink. On the day of the test, a fingernail-sized mid-dorsum patch was shaved in order to allow examination of the skin colour, and thus allow discrimination of the hair follicle cycling stage. The results were recorded as follows: "yes" if the skin was black, "no" if the skin was dark grey, grey or pink, and "non-synchronous" if the skin showed non-uniform colouration. The test was not performed if the mouse was albino or any other coat colour where the skin is non-pigmented (such a mouse was scored as an albino). In the case of agouti mice, any colour other than black was recorded as "no".

2.2.6.6 OPEN FIELD

This test was performed to measure locomotion, habituation and fear/anxiety responses to a novel environment. The test was conducted during the light period of the light-dark cycle. At least 5 minutes before the test, the light levels in the room were adjusted to 5 lux. All mice were individually tested for 10 minutes using the ActiMot2 surrounding an open field apparatus (TSE Systems) (**Figure 2.1**). At the beginning of the test, each

mouse was individually transferred to the arena, half way along the Y axis from a height of 5 cm with its tail parallel to the wall, and left undisturbed for the duration of the test. The anxiety and exploratory behaviour were recorded by camcorder for later analysis. Analysis was performed using ActiMot software for Windows (TSE Systems). The following parameters of the mice behaviour were recorded: total distance travelled (arena, periphery and centre), total resting time (arena, periphery and centre), total time (periphery and centre), average speed (arena, periphery and centre), total rears, latency to enter centre, number entries to centre, percentage distance moved in centre, and percentage time spent in centre. Average speed was calculated by dividing the total distance by the total time spent moving. The open field apparatus was cleaned with 70% ethanol after testing each mouse.

Figure 2.1. Photo of an ActiMot2 surrounding an open field apparatus. Photo taken from http://www.tse-systems.com/products/behavior/anxiety/open-field.htm.

2.2.6.7 SmithKline Beecham Pharmaceuticals; Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; Imperial College School of Medicine at St Mary's; Royal London Hospital, St Bartholomew's and the Royal London School of Medicine; Phenotype Assessment (SHIRPA)

This procedure was applied to assess for the presence of gross motor and neurological abnormalities. Mice were transferred to the test area 15 minutes before testing. After acclimatization, each mouse was first observed in the viewing jar (**Figure 2.2**) for 1 minute, and the following behaviours were recorded: body position (if the mouse was inactive, active or markedly active); palpebral closure; the presence or absence of lacrimation, tremor, defecation and urination. Subsequently, the mouse was transferred from the viewing jar to the centre of the arena (**Figure 2.3**) from approximately 30 cm above the arena floor and its behaviour was recorded for 30 seconds after it landed on the arena floor. The following parameters were scored: transfer arousal (the reaction to the new environment was recorded, namely if the mouse started to move instantly, froze briefly or froze for a period longer than a few seconds), gait (including ataxia), tail elevation (if during forward movement the tail was dragged, elevated or horizontally extended), pelvic elevation during forward movement, convulsions, head bobbing/circuling, locomotor activity (the number of squares that the mouse entered with all four feet during the total time spent in the arena), and touch escape (the mouse was approached from its front and subsequently the reaction of the mouse to touching the back of its neck was recorded). Also, whilst in the arena, the mouse was checked for its startle response after administering the sound stimulus from the click box (the stimulus was applied for the first time when the mouse stopped moving in the arena). Next, the mouse was lifted from the arena by the tail and suspended briefly to check for positional passivity (in other words to check if the mouse struggled when being held by the tail), trunk curl (to see if the mouse curled forward from its head towards its abdomen), and limb grasping. Finally, the mouse was placed on the metal grid on the top of the arena and checked for pinna touch reflex (the proximal part of the inner canthus was touched lightly with the tip of a fine cotton probe and the mouse's ear was observed for retraction), corneal touch reflex (the cornea was touched lightly with the tip of a fine cotton probe and the mouse's eye was

observed for the eye-blink response) and contact righting reflex (the mouse was placed in the 30 mm Perspex tube and the tube was slowly rotated by 180° to check whether or not the mouse attempted a righting reflex). During the whole SHIRPA test, the mouse was observed for evidence of biting and vocalization. All the equipment used was cleaned with 70% ethanol after testing each mouse.

Figure 2.2. Photo of a SHIRPA viewing jar. Photo taken from http://phenome.jax.org/db/q?rtn=projects/docstatic&doc=Lake2/Lake2_Protocol.

Figure 2.3. Photo of a SHIRPA arena. Photo taken from http://phenome.jax.org/db/q?rtn=projects/docstatic&doc=Lake2/Lake2_Protocol.

2.2.6.8 GRIP STRENGTH

A grip strength test was performed to assess the neuromuscular function and muscular strength of the fore and hind paws, and was recorded using a grip strength meter (BIO-GT3; Bioseb) (**Figure 2.4**)**.** The mouse was lowered towards the apparatus grid, being held only by its tail, until it gripped the grid with its fore paws only. When the tension of the grip was felt, the mouse was pulled back slowly by the tail (the mouse torso was kept horizontal to the grid) until the grip was released. Three trials of fore paws were performed immediately one after the other. Next, the mouse was lowered towards the apparatus grid, being held only by its tail, until it gripped the grid with both its fore and hind paws. When the tension of the grip was felt, the mouse was pulled back slowly by the tail (the mouse torso was kept parallel to the grid) until the grip was released. Three trials of all paws were performed immediately one after the other. At the end, an average value of both fore and fore/hind paw grip strength was calculated. The grip strength meter was cleaned with 70% ethanol after testing each mouse.

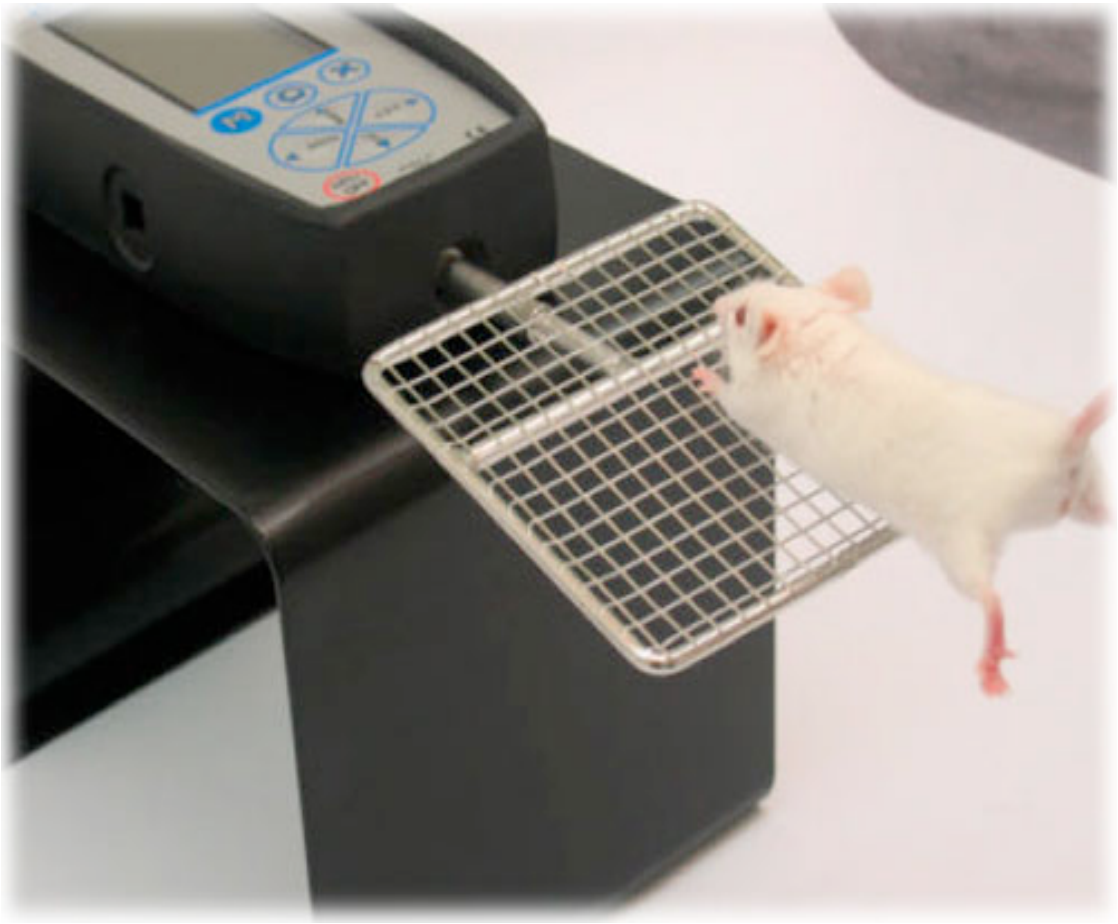

Figure 2.4. Photo of a grip strength meter. Photo taken from http://www.bioseb.com/bioseb/anglais/default/item_id=48_cat_id=2_Test%20d/item.php?mode=photo&id=62.

2.2.6.9 DYSMORPHOLOGY

Whole body morphology was examined to check for the presence of any abnormalities. First, the mouse was placed on the cage grid and checked for any obvious dysmorphologies in the physical appearance; irregularities in the shape of the body, head, tail, ears, and/or eyes; and irregularities in the coat colour, skin pigmentation, hair distribution and development. Next, the mouse was scruffed and observed for the presence or absence of whiskers; irregularities in the genitals; malformations of the ventral and lateral sides of head and body; shape, number and colour of the incisors; number and shape of digits; number, size and colour of paw pads; paw size and limb size; and irregularities in skin pigmentation on limbs.

2.2.6.10 HOT PLATE

This procedure was performed to assess for thermal pain perception. Each mouse was placed on a plate (TSE Systems) that had been heated to 52°C (**Figure 2.5**). The latency to the first response and the type of response were recorded. Mice that did not respond after 30 seconds were removed from the plate. The plate was cleaned with 70% ethanol after testing each mouse.

Figure 2.5. Photo of a hot plate meter. Photo taken from http://www.tse-systems.com/products/other-products/analgesia/hot-plate.htm.

2.2.6.11 INDIRECT CALORIMETRY

This experiment was performed to investigate the metabolism, circadian pattern and behaviour (mouse activity and to some extend its exploratory behaviour). Each mouse was weighed and then placed into a separate calorimetry cage (TSE Systems) with a handful of woodchips from its original cage (**Figure 2.6**). The reference cage was set up alongside cages

containing mice and the gas values were recorded from the reference cage an hour after the start of the experiment. The whole experiment lasted approximately 21 hours and consisted of a 5-hour light phase acclimatization period, followed by a 12-hour dark phase, and at least a 3-hour light phase. The following parameters were measured: cumulative food intake, activity (recorded as beam breaks), volume of oxygen consumed and volume of carbon dioxide produced. The respiratory exchange ratio and energy expenditure were derived from the above data. The water intake was assessed as the difference in water bottle weight before and after the experiment. When the experiment was finished, the mouse was returned to its original cage.

Figure 2.6. Photo of a calorimetry cage. Photo taken from http://www.tse-systems.com/download/TSE_Metabolism_LabMaster_PhenoMaster_20081014.pdf.

2.2.6.12 INTRA-PERITONEAL GLUCOSE TOLERANCE TEST (ipGTT)

This procedure was applied to obtain the fasted, basal blood glucose concentrations as well as to investigate glucose tolerance and clearance. Before the ipGTT, mice were fasted overnight for no more than 16 hours. The next day, each mouse was weighed and then a sample of blood was collected (representing 'fasting blood') before a bolus of 20% glucose was administrated by intra-peritoneal injection. Subsequently, blood samples were taken 15, 30, 60 and 120 minutes after the glucose administration. All blood samples were tested for their glucose concentration using Accu-Check® Aviva blood glucose meter (Roche) and Accu-Check® Aviva strips (Roche) modified such that there was no interference with maltose. Blood samples were taken from the tail. The tip of the tail was cut using a blade. The blood was brought to the tip of the tail by running two fingers laterally up the mouse's tail from the base of the tail. The dripping blood was applied to the yellow tip of an Accu-Check® Aviva strip (the other end of the strip was inserted into the Accu-Check® Aviva blood glucose meter), and the blood glucose concentration (in mmol/l) was read from the meter's screen.

2.2.6.13 AUDITORY BRAINSTEM RESPONSE (ABR)

This procedure was performed to assess the hearing sensitivity across a broad range of frequencies. Each mouse was first tested with a click box to check for the presence of the Preyer Reflex, then anaesthetized with ketamine/xylazine by intraperitoneal injection (as described in section 2.2.6.1) and placed on a heating blanket in a sound chamber. After anaesthesia was confirmed, sub-dermal needle electrodes were inserted (active electrode on vertex, reference electrode overlying left bulla, and ground electrode overlying right bulla). The mouse was placed lying in a prone position with paws forward, facing the loudspeaker at a distance of 20 cm and an initial click-evoked ABR (256 clicks @ 70 dB Sound Pressure Level (SPL)) was recorded from the mouse's scalp to ensure a good ABR was present. Then, ABRs were recorded at the following frequencies and levels: 6 kHz (20 – 85 dB), 12 kHz (0 – 70 dB), 18 kHz (0 – 70 dB), 24 kHz (10 – 70 dB) and 30 kHz (20 – 85 dB), presented in 5 dB intervals. At the end, a final

click-evoked ABR (e.g. 256 clicks at 70 dB SPL) was recorded. Subsequently, the electrodes were carefully removed and anaesthesia was reversed using antisedan (as described in section 2.2.6.1), and the mouse was returned to its original cage.

2.2.6.14 DUAL ENERGY X-RAY ABSORPTIOMETRY (DEXA)

This procedure was applied to obtain comprehensive body composition and bone mineral data. The mouse was either anaesthetised (ketamine/xylazine) (as described in section 2.2.6.1) or euthanized by asphyxiation with rising levels of carbon dioxide. Then, the body weight and length were measured, and X-ray images were collected using a Lunar PIXImus II Bone Densitometer (GE Medical Systems) (**Figure 2.7**). To do this, the mouse was placed ventrally onto the white sticky DEXA tray (**Figure 2.7**). The head of the mouse was placed to the left with its snout in the holding slot. The paws were placed away from the body with pads down. The tail was curled to the left around the body so it was entirely within the scanning zone but such that it did not obstruct any other body parts. The mouse was then subjected to the DEXA scan. When all X-ray images were taken, the anaesthesia was reversed using antisedan (if applicable) (as described in section 2.2.6.1). The PIXImus software package generated the image of the entire mouse and provided bone mineral and body composition data. The PIXImus software package also automatically analysed the resulting images, excluding the skull, to calculate total tissue mass, body fat mass, lean mass, fat percentage estimate, bone area, bone mineral density (BMD), and bone mineral content (BMC). Quality control measurements using a ‘phantom’ mouse were performed before each imaging session.

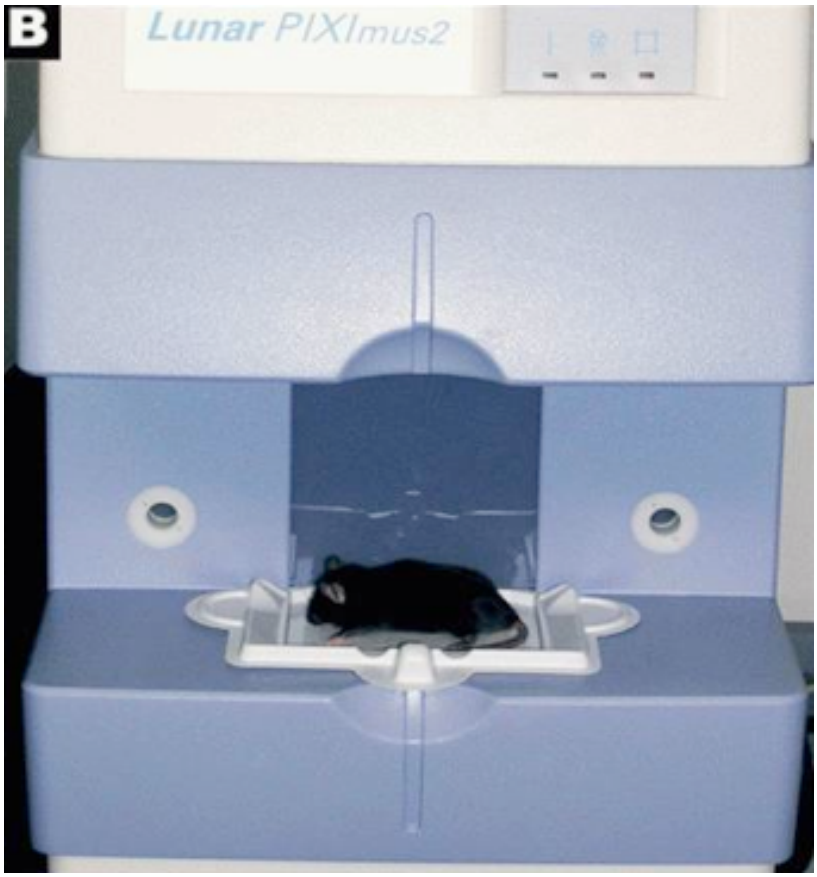

Figure 2.7. Photo of a Lunar PIXImus II Bone Densitometer. Photo taken from http://phenome.jax.org/db/q?rtn=projects/docstatic&doc=Donahue1/Donahue1_Protocol.

2.2.6.15 X-RAY IMAGING

This procedure was conducted to obtain high-resolution X-ray images of the mouse skeleton. X-ray scans were taken using a Faxitron system MX20 (Faxitron X-ray Coorporation). The mouse was either anaesthetised (ketamine/xylazine) (as described in section 2.2.6.1) or euthanized by asphyxiation with rising levels of carbon dioxide, and then five X-ray images acquired, specifically whole body (dorso-ventral and lateral), head (dorso-ventral and lateral) and left forepaw (dorso-ventral). In order to take the dorso-ventral X-ray image of the whole body, the mouse was arranged ventrally in the image zone with its head pointing towards the upper left corner of the zone and its rear towards the lower right corner with the red crosshair on its back. The paws were placed away from the body with the pads down. The tail was curled to the left around the body so it did not cover any other

body parts but fitted completely into the X-ray zone. In order to take the lateral X-ray image of the whole body, the mouse was placed on its side in the image zone with its head pointing towards the upper left corner of the zone and its rear towards the lower right corner with the red crosshair on its rump. The right hind paw was pulled out horizontally and taped perpendicular to the surface. The left hind paw was pulled out and taped to the surface to make it look as natural as possible. The tail was curled behind the mouse's body and along its spine so it did not cover any other body parts but fitted completely into the X-ray zone. In order to take the dorso-ventral X-ray image of the head, the mouse was arranged ventrally in the image zone with its head in the centre. The head was placed in such a way that the red crosshair was on the top of its head just above the ears. In order to take the lateral X-ray image of the head, the mouse was placed on its side onto the image zone with its head in the centre. The head was placed in such a way that the vertical red crosshair was between its eye and ear, and the horizontal crosshair was along its mouth or snout. In order to take the dorso-ventral X-ray image of the left fore paw, the mouse was arranged ventrally in the image zone with its left forelimb in the lower left quadrant of the zone and with the fore paw being in the upper left quadrant. The mouse was placed in such a way that the red crosshair was just below the ear of the mouse. When all X-ray images were taken, the anaesthesia was reversed using antisedan (if applicable) (as described in section 2.2.6.1). The following parameters/abnormalities were recorded: skull shape; morphology of zygomatic bone, maxilla, mandible, teeth, scapula, clavicle, humerus, radius, ulna, femur, tibia, fibula, pelvis and joints; number, shape and fusion of ribs; shape of ribcage and spine; presence or absence of scoliosis, kyphosis or lordosis; number of cervical, thoracic, lumbar, caudal and pelvic vertebrae; transitional vertebrae; shape and fusion of vertebrae; processes on vertebrae and spinous; transverse processes; fusion of processes; number of digits; digital integrity; and presence or absence of polysyndactylism, brachydactylism or syndactylism.

2.2.6.16 CORE TEMPERATURE STRESS

This procedure was performed to determine the basal body temperature and to assess stress-induced hyperthermia. The basal core body

temperature was measured rectally using a TH-5 thermometer with a RET-3 probe (Viking Medical). Mice were transferred to the test area 1 hour before testing, and after acclimatization they were placed on the wire grid of the clean cage. The hind-end of the mouse was raised (the mouse was held by the base of its tail), letting the mouse grip the grid with only its fore paw. Next, using the middle finger, pressure was applied onto the lumbar spinal region of the mouse to arch its back, and the probe was gently introduced into the mouse rectum. After the measurement was taken, the mouse was placed in a clean cage and left there for 15 – 30 minutes. Thereafter, a second reading was taken before the mouse was returned to its original cage.

2.2.6.17 EYE MORPHOLOGY SCREEN

This procedure was performed to detect gross morphological abnormalities in the eye. Both eyes were assessed for the presence of any morphological changes using a slit lamp (Zeiss SL130) and ophthalmoscope (Heine Omega 500). Images on the slit lamp were collected using a LEICA DFC420. Images of the fundus were collected using a topical endoscope (BERCI Tele-Otoscope with HOPKINS straight forward 0°, diameter 3 mm, Halogen cold light fountain light source) and camera (Nikon D40x with Nikon AF 85 mm F1.8D AF Nikkor lens). During the examination, the mouse was first observed for general abnormalities in eye general morphology and size, eye bulging and eyelid closure. Next, both eyes were examined when undilated using the slit lamp. The following parameters/abnormalities were recorded: presence of blood in or around the eye; discharge in or around the eye; corneal morphology, opacity, vascularisation and mineralization; lens stalk; pupil shape, position, dilation and light response (to test this response, the light was dimmed for a few seconds and then brought to its original level); and iris position and pigmentation. Subsequently, both eyes were examined when dilated (induced with 1% tropicamide and/or neosynephrine) using the slit lamp. The following parameters were recorded: lens morphology and opacity (non suture or snowflake), corneal irregularities, and synechia. Finally, both eyes were examined in a dilated state using an ophthalmoscope. The following parameters/abnormalities were recorded: retina morphology,

structure and pigmentation; blood vessel morphology pattern, number and structure; optic disc morphology; Bergmeister's papilla morphology; and whether a cataract was visible.

2.2.6.18 RETRO-ORBITAL BLEED

This procedure was applied to obtain high-quality blood samples from the retro-orbital sinus via a capillary tube. Each mouse was anaesthetized with ketamine/xylazine by intraperitoneal injection (as described in section 2.2.6.1). After anaesthesia was confirmed, the capillary tube was inserted into the space between the globe and the lower eyelid at an approximately 45° angle. Next, the capillary was directed gently in a ventrolateral direction while rotating the capillary tube, and the flowing blood was collected into the capillary tube. From each mouse, two collection tubes were filled with dripping blood. 50 μl of blood was collected into an EDTA-coated tube (Kabe Labortechnik) for subsequent haematological analysis (this tube was kept on ice), while 1 ml of blood was collected into a lithium heparin-coated tube (Kabe Labortechnik) for subsequent clinical chemistry analysis (this tube was kept at room temperature). After the blood was collected, the mouse was culled by cervical dislocation.

2.2.6.19 HEART WEIGHTS

This procedure was performed to assess the weight of the mouse's heart. Each mouse was terminally anaesthetized with ketamine/xylazine by intraperitoneal injection (as described in section 2.2.6.1). After anaesthesia was confirmed, its heart was dissected out and weighed before being placed in a 10% neutral buffered formalin for subsequent histopathological analysis.

2.2.6.20 HAEMATOLOGY PANEL

This procedure was performed to analyse the following whole blood parameters: white and red blood cell count; mean corpuscular volume; haemoglobin; erythrocyte indices (hematocrit, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, and red blood cell distribution width); platelet count; and mean platelet volume. The

non-fasted mouse was terminally anaesthetised with ketamine/xylazine and 50 μl of blood was collected into the EDTA-coated tube (Kabe Labortechnik) by retro-orbital bleeding. All blood samples were automatically analysed using a haematology analyser (scil Vet animal blood counter, Horiba Medical, cat. #: RS 232).

2.2.6.21 PLASMA CHEMISTRY PANEL

This procedure was performed to analyse the following parameters from the blood plasma: sodium, potassium, chloride, glucose, triglycerides, cholesterol, high density lipoprotein, low density lipoprotein, non-esterified free fatty acids (NEFAC), glycerol, amylase, alanine aminotransferase, alkaline phosphatase, creatine kinase, aspartate aminotransferase, total bilirubin, total protein, albumin, creatinine, urea, calcium, magnesium, iron, phosphatase, lactate dehydrogenase, and uric acid. The non-fasted mouse was terminally anaesthatised with ketamine/xylazine and 1 ml of blood was collected into a lithium heparin-coated tube (Kabe Labortechnik) by retro-orbital bleeding. All plasma samples were automatically analysed in an Olympus AU400 chemistry immunoanalyser (Alternative Biomedical Solutions).

2.2.6.22 BEHAVIOURAL TESTS

These tests used male monosomic *Df*$^{Lipi\text{-}Usp25}$ mice and male wildtype littermate controls fed on a normal-fat diet who were 3- to 7-month old and were group-housed from weaning. All mice were prehandled for 1 – 2 minutes every day for four days prior to testing for habituation.

2.2.6.22.1 ELEVATED PLUS MAZE

This test was performed to assess anxiety responses to a novel environment. The elevated plus maze was made of Plexiglas and homogeneously illuminated with tests performed under dim light. The apparatus consisted of a central square and four 30 cm long and 5 cm wide black arms, of which two were closed by 15 cm high walls and two were open (**Figure 2.8**). The maze was elevated 40 cm off the floor (**Figure 2.8**). The

test was conducted during the light period of the light-dark cycle. At least 5 minutes before the test, the light levels in the room were adjusted to medium. Each mouse was placed in the centre square in such way that it faced both towards the open arm and away from the experimenter, and left to explore the maze for a single 5-minute session with the experimenter out of view. Each mouse was tested once. When the test was finished, the mouse was put back into its original cage, and the maze was cleaned with 70% ethanol before testing another mouse. All test sessions were recorded and subsequently analysed with the Noldus Ethovision 3 video tracking software (Tracksys Ltd., UK). The tracking system recorded time spent in each arm and number of entries into each arm, with an animal being considered inside a zone whenever its centre point lay within.

Figure 2.8. Photo of an elevated plus maze. Photo taken from http://www.med-associates.com/mazes/elevated-maze.htm).

2.2.6.22.2 SOCIAL RECOGNITION TEST

This test was performed to assess 24-hour social memory retention. The test was performed under red light, using 3 different 2-month old male stimulus mice (C57BL/6NTac/USA (mouse A), 129P2/OlaHsd (mouse B) and C57BL/6NTac/USA (mouse C)) sedated with ketamine/xylazine (i.p. 1 g/0.1 g per kg of body weight). Test mice were first habituated for 10 minutes to a test arena identical to their home cage. On day 1, for the habituation-dishabituation test, a stimulus mouse A was placed into the test arena for 1 minute. The same stimulus mouse A was subsequently presented four times at 10 minutes intervals. In the fifth trial, a stimulus mouse B was presented for 1 minute. On day 2, for the discrimination test, the animals were simultaneously presented with stimulus mouse A (familiar animal that was encountered on trials 1 – 4 on day 1) and mouse C (new unfamiliar animal) for 2 minutes. All trials were recorded with an overhead camera and the videos were subsequently scored blind of genotype using a handheld stopwatch. The amount of time the test animal spent investigating, by direct oronasal contact or close approach (about 1 cm), sniffing towards the stimulus mice A and C, was recorded. If the test animal spent longer investigating a novel stimulus animal (mouse C) than the familiar one (mouse A), this was taken as evidence for social recognition. The discrimination ratio was taken as the amount of time spent investigating the familiar stimulus animal (mouse A) divided by the sum of time spent investigating both stimulus mice A and C. These data are presented as a mean and a standard error in the mean, and were analysed using GraphPad Prism software (GraphPad Software Inc.). Statistical analysis for the habituation-dishabituation test data was performed using a two-way ANOVA with repeated measures for trial and genotype as factor and a Tukey-HSD *post hoc* test. Discrimination data was analysed using the two-tailed Student's *t*-test based on the discrimination ratio.

2.2.6.23 INFECTIONS OF MICE WITH BACTERIA

2.2.6.23.1 *CITROBACTER RODENTIUM* INFECTION – AN OVERVIEW

An equal number of female wildtype and mutant mice were infected with

Citrobacter rodentium lux when they were 6 weeks of age (see section 2.2.6.23.1.2 below for details). Mice were monitored and weighed daily for 28 days post-infection. Faecal samples from each mouse were collected on day 1 post-infection, and then every 2 – 3 days until day 27 post-infection (see section 2.2.6.23.3.2 below for details). The amount of bacteria in faecal samples was enumerated by serial dilution and plating onto agar plates (see section 2.2.6.23.4 below for details). On day 14 post-infection, half of the mice (of each genotype) were culled by cervical dislocation, and spleens, livers, caecal patches, caecums and at least 6 cm of distal colon, and caecal contents were extracted for bacterial counts (see section 2.2.6.23.3.2 below for details). The amount of bacteria in collected tissues/ceacal contents was enumerated by serial dilution and plating onto agar plates (see section 2.2.6.23.4 below for details). The 6 cm of distal colon was weighed, checked for signs of gross hyperplasia, and a 5 mm section from the most distal colon was removed and placed into 10% neutral buffered formalin for subsequent histopathological analysis. All these analyses were repeated on the remaining mice at day 28 post-infection.

2.2.6.23.1.1 PREPARING INOCULUM TO INFECT MICE WITH *C. RODENTIUM*

One cryo-vial of *Citrobacter rodentium* lux was put into a conical flask containing 100 ml of LB with kanamycin (50 mg/ml) and naladixic acid (50 mg/ml). The flask was incubated in a shaking incubator at 37°C overnight. The next morning, 20 ml of the overnight culture was added to a 50 ml Falcon tube and centrifuged at 40,000 rpm for 10 minutes. Next, the supernatant was removed and the pellet resuspended in 10 ml of PBS. This was called the 'inoculum'.

2.2.6.23.1.2 INFECTING MICE WITH *C. RODENTIUM*

1.5 ml of the inoculum was drawn up into a syringe with the gavage needle. Once the air bubbles had floated to the top, the volume of the syringe was adjusted so there was 1 ml of inoculum. Prior to the infection, a mouse was anaesthetized using 100% isoflurane gas, IsoFlo (Abbott, cat. #: 05260-05). Next, the gavage needle was gently slid down the

oesophagus and into the stomach of the anaesthetized mouse that was being held in the scruffed position. The plunger was slowly depressed and 0.2 ml of the inoculum injected into the stomach. The gavage needle was then gently withdrawn and the mouse put back into its original cage and closely observed util it recovered.

2.2.6.23.2 *SALMONELLA* TYMPHIMURIUM INFECTION – AN OVERVIEW

An equal number of male control and mutant mice were infected with *Salmonella* Typhimurium TET C when they were 6 weeks of age (see section 2.2.6.23.2.2 below for details). Mice were monitored and weighed daily for 28 days post-infection. On day 14 post-infection, half of the mice (of both genotypes) were culled by cervical dislocation and the spleens, livers and caecal contents were extracted for bacterial enumeration (see section 2.2.6.23.3.2 below for details). The amount of bacteria was enumerated by serial dilution and plating onto agar plates (see section 2.2.6.23.4 below for details). Additionally, 1/4 of the spleen and one lobe of the liver were dissected from each mouse, and placed into 10% neutral buffered formalin for subsequent histopathological analysis. On day 28 post-infection, the remaining mice were terminally anaesthatised with ketamine/xylazine, bled via cardiac puncture to obtain blood serum for testing for anti TET C specific antibodies by enzyme-linked immunosorbent assay (ELISA) (IgG, IgG1 and IgG2a) (see section 2.2.6.23.6 below for details), and then analysed in the same way as the day 14 post-infection mice.

2.2.6.23.2.1 PREPARING INOCULUM TO INFECT MICE WITH *S.* TYPHIMURIUM

100 μl of *Salmonella* Typhimurium M525 TET C was added to a universal tube containing 10 ml of PBS and then gently vortexed. Next, 100 μl of its content were added into another universal tube containing 10 ml of PBS and gently vortexed. This was called the ‘inoculum’.

2.2.6.23.2.2 INFECTING MICE WITH *S.* TYPHIMURIUM

Prior to infection with *Salmonella* Typhimurium, the mouse was put into a

heat chamber box (Harvard Apparatus UK, cat. #: IC016000) set at 37ºC for 20 to 30 minutes. 1.5 ml of the inoculum was drawn up into a syringe and, once the air bubbles had floated to the top, the volume of the syringe was adjusted so there was 1 ml of inoculum. Then the mouse was removed from the heat chamber box and placed in a restraining tube. The needle was gently inserted into a tail vein and the plunger slowly depressed util 0.2 ml of the inoculum had been injected into the vein. Finally, the needle was gently withdrawn and the mouse returned to its original cage.

2.2.6.23.3 PROCESSING OF BLOOD FOR SERUM AND TISSUES, CAECAL CONTENTS AND FAECAL SAMPLES FOR BACTERIAL COUNTS FROM MICE INFECTED WITH *C. RODENTIUM* OR *S.* TYMPHIMURIUM

2.2.6.23.3.1 BLOOD

Blood tubs were centrifuged at 13,000 rpm for 5 minutes. The top layer of serum was transferred into a fresh eppendorf tube and stored at -20°C.

2.2.6.23.3.2 TISSUES/FAECAL CONTENTS

Tissues/caecal contents were collected into plastic bags, which were then weighed. The contents were then thoroughly broken up by hitting them with the lid part of a falcon tube and 5 ml of ddH_2O added into each bag. The bag was left at room temperature for 10 minutes before being placed in the Stomacher machine (Seward) set at the highest speed for 2 minutes. The resulting liquid content was used to prepare the serial dilutions for bacterial enumeration.

2.2.6.23.3.3 FAECAL SAMPLES

Faecal samples were collected into eppendorf tubes, which were then weighed. Next, 100 μl of PBS was added for every 0.01 g of faecal sample and the whole content was vortexed for about 5 minutes until the faecal sample was fully homogenized. Then the eppendorf tube was centrifuged at 13,000 rpm for 1 minute and the liquid content used to prepare the serial dilutions for bacterial enumeration.

2.2.6.23.4 BACTERIA ENUMERATION IN TISSUES, CAECAL CONTENTS AND FAECAL SAMPLES

To count the amount of bacteria in collected tissues, caecal contents or faecal samples, a serial dilution of each sample was prepared, which was then plated onto agar plates. Specifically, five 10-fold dilutions were prepared in a 96-well plate for each sample (1:1, 1:10, 1:100, 1:1000, 1:10000, 1:100000 dilutions of the sample). To do this, 200 μl of sample (liquid content) either from the bag (tissues or caecal contents) or eppendorf tube (feacal samples) was placed into well 1A of a 96-well plate (this represents the neat (N) concentration). Next, the contents of well 1A was pipetted up and down a few times and 50 μl was transferred into well 3A well containing 450 μl of ddH_2O (for tissues or caecal contents) or 450 μl of PBS (for feacal samples) to obtain a 1:10 dilution. This process was repeated in the same way in order to transfer 50 μl of the content from well 3A to 5A, 5A to 7A, 7A to 9A, and 9A to 11A to obtain 1:100, 1:1000, 1:10000, 1:100000 dilutions respectively. The tips were changed after each transfer. Next, 20 μl of dilution from wells 1A, 3A, 5A, 7A, 9A, and 11A was pipetted up and down a few times and plated on an LB agar plate containing kanamycin (50 mg/ml) and naladixic acid (50 mg/ml). Once dry, the plates were inverted and put in an incubator at 37°C overnight.

2.2.6.23.5 COUNTING THE BACTERIAL COLONIES FROM TISSUES, CAECAL CONTENTS AND FAECAL SAMPLES

LB agar plates were removed from the incubator after being left overnight at 37°C. Plates were placed against a light surface such that the lids were facing downwards. The colonies were counted by hand with a marker pen that was used for marking each individual colony.

2.2.6.23.6 DETERMINATION OF IMMUNOGLOBULIN TITRE FROM BLOOD SERUM

Each well of the Nunc MaxiSorp™ flat-bottom 96 well plate (eBioscience, cat. #: 44-2404-21) was coated with 50 μl of 2 mg/ml TET C protein (antigen) solution in coating buffer (0.1 M Na_2HPO_4; pH 9). Three

plates were prepared at the same time. Plates were then sealed and incubated at 4°C overnight. The next day, the coating solution was flicked out of the plates and 150 μl of blocking buffer (3% Bovine Serum Albumin (BSA) in 1x PBS, kept on ice) was added to each well. Plates were then sealed and incubated at room temperature for 1 hour. After incubation, the blocking buffer was flicked out of all plates and the plates were rinsed with wash buffer (0.05% Tween-20 in 1x PBS). Next, 112.5 μl of antibody buffer (1% BSA in 1x PBS, kept on ice) was added to the top row of wells and 100 μl of the buffer was added to the remaining wells. In a fresh eppendorf tube, 6 μl of serum (containing the primary antibody) from 4 control and 4 mutant mice was added to 54 μl of antibody buffer. Then 12.5 μl of the diluted serum was added to the top row of wells of each plate. The order of serums was as follows: mutant M1, M2, M3 and M4, 2 blank wells, wildtype W1, W2, W3 and W4, and 2 blank wells. Next, the serums were serially diluted down each plate. The serums in the top row of wells were mixed by pipetting a few times, then 25 μl was transferred to the second row. This process was repeated all the way down the plate (i.e. all 8 rows) and tips were changed after each transfer. Then the plates were sealed and incubated at 37°C for 1 hour. After incubation, the diluted serum was flicked out of the plates, which were then washed three times with wash buffer for 5 minutes. Then 100 μl of the HRP-conjugated secondary antibody (either Ig, IgG1 or IgG2a) was added to the plate (secondary antibodies were diluted 1:1000 in antibody buffer). The plates were sealed and incubated at 37°C for 1 hour. The secondary antibody was then flicked out of the plates, which were washed three times with the wash buffer for 5 minutes. Then *o*-phenylenediamine tablets (Sigmafast tablet set; Sigma-Aldrich, cat#: P9187) were added to 20 ml of ddH_2O in a 50 ml Falcon tube and kept on ice. The ELISA reaction was developed by adding 50 μl of *o*-phenylenediamine mixture to each well of the plate. Plates were then sealed and incubated at room temperature for 15 minutes. After the wells in the top row had turned brown, the reactions were terminated by the addition of 20 μl of stop solution (12.5% sulphuric acid) to each well. The absorbance of the plate was read using the benchmark plate reader at an optical density (OD) of 490 nm.

2.2.7 TUMOUR WATCH STUDY

Blm$^{m3/m3}$ homozygous knockout mice (Luo 2000) were provided by Professor Allan Bradley (The Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK). The *Blm*$^{m3/m3}$ mice were crossed with the *Df*$^{Lipi\text{-}Usp25}$*/+ mice* to generate a cohort of mice for tumour watch study. From all obtained mice, only mice with the following genotypes were used in the study: 1) *Df*$^{Lipi\text{-}Usp25}$*/+, Blm*$^{m3/m3}$, 2) *Df*$^{Lipi\text{-}Usp25}$*/+, Blm*$^{+/+}$, 3) *+/+, Blm*$^{m3/m3}$. Mice on tumour watch were observed twice a day for signs of illness/morbidity, at which time a full necropsy was performed and all tissues macroscpically examined before being placed in 10% neutral buffered formalin for histopathological analysis.