Chapter 2: Materials and Methods

All solutions used are listed at the end of the chapter.

All chemicals were obtained from Sigma unless otherwise stated.

2.1 Centrifuges

Listed below are the centrifuges used at various stages of this project:

- Eppendorf Centrifuge 5417R – used for all protocols unless otherwise stated
- Eppendorf Centrifuge 5702 – ES Cell culture (section 2.4), Optimised EB formation protocol (section 2.5.3) and Cell cycle assay: Cell Growth (section 2.5.4.1)
- Thermo Electron Heraeus Sepatech Megafuge 1.0 – Fluorescence activated cell sorting (section 2.5.4.2) and Harvesting HeLa S3 RNA (section 2.7.4)
- Beckman J6-MC – Trizol RNA purification of large volumes in 50ml Falcon tubes (section 2.7.1)
- Thermo Electron Heraeus Pico 21 – Preparing Illumina/Solexa samples (section 2.10.3.1) and Northern blot preparation (section 2.9.1.1)
- Eppendorf Plate Centrifuge 5403 – KIF11 siRNA transfection (section 2.12.2)

2.2 General techniques

2.2.1 Phenol chloroform extraction

0.5 vol. phenol and 0.5 vol. chloroform (24 chloroform : 1 isoamylalcohol) were added to the DNA. The tube was mixed by shaking and spun at maximum for 15 minutes to separate phases. The aqueous phase was removed to a fresh tube and an

equal volume of chloroform (24 chloroform : 1 isoamylalcohol) was added. The solution was mixed by shaking and the phases were separated by spinning for 5 minutes. The aqueous layer was removed to a separate tube and ethanol precipitated (see section (2.2.2)).

2.2.2 DNA ethanol precipitation

2 volumes of ice cold 100% ethanol and 1/10 volume of 3 M sodium acetate were added to the DNA in aqueous solution. The solution was mixed by inversion and placed at -20˚C overnight. The DNA was then pelleted by spinning at maximum for 30 minutes at 4˚C. The DNA was washed with >500 µl of 70%-75% ethanol and spun for 5 minutes at 4˚C. Finally the pellet was air dried and resuspended in $T_{0.1}E$.

2.2.3 Small RNA isopropanol precipitation

An equal volume of isopropanol was added to the RNA sample and 3 µl of GlycoBlue (Ambion). The contents were mixed by inversion and placed on ice for 30 minutes. The spin and washes were conducted following the ethanol precipitation protocol (see section 2.2.2), but using RNase free solutions. The pellet was resuspended in RNase free water.

2.2.4 PCR

Standard polymerase chain reaction (PCR) was conducted using KOD Hotstart Polymerase (Novagen) in either 25 µl (2.5 µl KOD Buffer, 2.5 µl 2 mM dNTPs, 1 µl 25 mM $MgSO_4$, 8.3 µl DDW + Template, 8.2 µl 3x Sucrose/Cresol Solution, 1 µl 15 µM Primer 1, 1 µl 15 µM Primer 2, 0.5 µl KOD Polymerase) or 15 µl reactions (1.5 µl KOD Buffer, 1.5 µl 2 mM dNTPs, 0.6 µl 25 mM $MgSO_4$, 4.9 µl DDW + Template,

5 µl 3x Sucrose/Cresol Solution, 0.6 µl 15 µM Primer 1, 0.6 µl 15 µM Primer 2, 0.3 µl KOD Polymerase) using a Peltier Thermal Cycler. If stated, the Expand 20kb PLUS PCR System (Roche) was used to amplify long PCR products, according to the manufacturers instructions, with the MEXP62 PCR programme (see section 2.2.4.2).

2.2.4.1 Primers

Primers were designed using the Primer3 programme (http://frodo.wi.mit.edu/) unless otherwise stated.

Number	Sequence	Pre-designed?
1	TACGGATCTGGAACTGCAAG	
2	ATTCAGGCTGCGCAACTGTTGGG	
3	CTCAAGGTCCGCCCTGTTTA	
4	AGTATCGGCCTCAGGAAGATCG	
5	AGGCACTCATGGAGGATCTG	
6	GTCACCCATTCCATGGTTTC	
7	GAGCTGGATGGAGCTGTAGG	
8	CGCCACCTTCAAAAGTTGTT	
9	CATTTGGGGACTCCTTGATG	
10	GTGACCTGGCCAGTAGACCA	
11	AATTGGCGCGCCCCTGGAGTAGGCATGTTGATTTCAC	
12	AATTGGCGCGCCATGCTGAGACAAGACTGGAAACCAC	
13	GAAGGTCTCTGTGCTCCCAAG	
14	CAAGATCTGAATCTGGGTGGTG	
15	AGAGAAGTGTGGTGCAGGTG	
16	GGGTGAGATCAAGGCTTCC	
17	GCGGATAACAATTTCACACAGGA	pUCR
18	TGTAAAACGACGGCCAGT	M13 Forward 1
19	GGAGAAAGGCGGACAGGTAT	
20	CCTTGAAGGACTCCAATAGGG	
21	GCTGCAGGAGTAAGGACAGG	
22	GTGGATGAAGAGGCCTTGAA	
23	TTCTTTGGTTTTCGGGACC	
24	GTTTTCGGGACCTGGGAC	
25	GCTGGGCTGTTGTCTCCATA	
26	CATCTTGGGTTTCTTCCGAGT	
27	CGACGACCCATTCAACTTCT	
28	TCGAGCACTGCATACTCCAC	
29	GTAAAACGACGGCCAGT	M13 Forward 2
30	GGAAACAGCTATGACCATG	M13 Reverse

Table 2.1: Primers used in during the course of this study

2.2.4.2 PCR programmes

MKOD6030

1. 94˚C – 2 minutes

2. 94˚C – 15 seconds

3. 60˚C – 30 seconds

4. 72˚C – 1 minute

Repeat steps 2-4 30x

5. 72˚C – 5 minutes

MEXP62

1. 92˚C – 2 minutes

2. 92˚C – 10 seconds

3. 62˚C – 30 seconds

4. 68˚C – 10 minutes

Repeat steps 2-4 10x

5. 92˚C – 10 seconds

6. 62˚C – 30 seconds

7. 68˚C – 10 minutes and 10 seconds + 10 seconds/cycle

Repeat steps 5-7 24x

8. 68˚C – 7 minutes

2.2.5 Colony PCR

A bacterial colony or ice scrape was picked into 100 µl DDW. 1µl of this solution was then used as the PCR template. A 15 µl or 25 µl KOD Hot-start (Novagen) PCR

reaction was then conducted with the MKOD6030 PCR programme. Products were subsequently size separated by gel electrophoresis (see section 2.2.6).

2.2.6 Gel electrophoresis

Agarose gel electrophoresis was used to size separate nucleic acids. 0.6%-2% agarose gels were prepared with 1x TBE containing ethidium bromide (400 ng/ml). Samples were loaded in 1x sucrose / cresol solution. Generally samples were loaded alongside a 1kb size marker (Invitrogen #15615-024). Mini-gels (50 ml) and larger gels (250 ml) were run at a voltage and for a period that would allow suitable size separation of nucleic acids. Nucleic acids were visualised with UV light on a transilluminator (UVP) and digital images were captured with a UVP system.

2.2.7 Bacterial culture

MACH1 *E. coli* (Invitrogen) cells, made competent by the rubidium chloride method, were kindly provided by James Grinham. Bacteria were cultured in Luria-Bertani (LB) broth or on LB agar plates supplemented with appropriate antibiotics (see below) at 37˚C. Liquid cultures were incubated in a shaking incubator (37˚C, 300 rpm). Blue/white selective plates were made by supplementing 500 ml of LB agar with 40 ng Xgal dissolved in 800 µl Dimethyl formamide and 2.5 ml 0.1 M Isopropyl ß-D-1-thiogalactopyranoside (IPTG). Bacteria were stored frozen at -70˚C in LB supplemented with 7.5% glycerol.

2.2.7.1 Antibiotic concentrations

Ampicillin: Final concentration of 100 µg/ml

Chloramphenicol: Final concentration of 30 µg/ml

Kanamycin: Final concentration of 50 µg/ml

2.2.7.2 Bacterial transformation

0.5 µl of plasmid was added to a frozen Thermowell 96-well plate (Costar), rested in a Stratacooler cooling block (Stratagene), and allowed to cool. Cells were defrosted on ice and 10 µl of MACH1 cells (Invitrogen) was added to each well containing plasmid. The plate and block were incubated in a Stratacooler (Stratagene) for 20 minutes. The cells were subsequently heat shocked for 40 seconds at 42˚C (Peltier thermal cycler) and returned to the ice box for a further 2 minutes. Finally the cells were removed from the ice box and 90 µl of LB was added to each well. The cells were placed in a 37˚C incubator for 1.5 hours and then the total volume was plated to warmed agar plates.

2.2.8 Sequencing

PCR fragments purified with the Qiagen Qiaquick Gel Extraction kit were diluted 1/5 -1/10 in Double distilled water (DDW) and 1µl of this template was added to 1µl of the appropriate primer (5µM) and 5µl of DDW. For plasmid sequencing 200 ng of plasmids purified by Qiagen Qiaprep Spin Miniprep were added to ~20 ng of the appropriate primer and the volume of the mix was adjusted to 7µl with DDW. These reaction mixtures were passed to the Sanger Core Sequencing Facility for sequencing. Gap4 (Staden Sequence Analysis Package software) was used to compile the sequencing results and join matching sequences. Basic Logical Alignment Search Tool (BLASTN) was used to compare consensus sequence against the mouse genome in Ensembl (http://www.ensembl.org/) or the Gap4 database was used to compare the sequences against an expected sequence based on Ensembl annotation

(ENSMUST00000009321, ENSMUST00000115633 (*Dgcr8*), ENSMUST00000023292 (*Arsa*)) and plasmid sequences.

2.2.9 Restriction digest

Restriction enzymes were supplied by NEB. Buffers were diluted as appropriate for the enzyme in question and bovine serum albumin (BSA) was added as applicable. Reaction volume varied as noted in individual protocols. On occasion 1/3 vol. of Sucrose/Cresol Solution was added to each reaction. Unless otherwise stated, 3 µl of restriction enzyme was added to each reaction, the reaction was incubated for 1 hour at 37˚C and a further 3µl of enzyme was added followed by a further incubation. Reactions were heat inactivated at 65-70˚C for 20 minutes and stored frozen. Digested plasmid was size separated by gel electrophoresis alongside non-digested plasmid to ensure the digest had reached completion (section 2.2.6). The digest was repeated if necessary.

2.3 Generating a targeting trap vector

2.3.1 Long PCR

129Sv strain derived bacterial artificial chromosomes (BACs) (Adams et al., 2005) within the *Dgcr8* region (judged by an Ensembl mapped distributed annotation system (DAS) trace (v33, m34)) were selected. Where the sequences were obtainable from the sequence repository (nfs/repository/p305/MSE-WG-58230), BLASTN was used to remap the end sequences against the Ensembl database to check their annotated location. BACs were obtained from the Sanger Clone Resource Centre. The BAC plasmid was purified using the Qiagen Quiaprep Spin Miniprep. ~800ng of BAC PCR templates were digested by *NotI* (NEB) in a 200µl reaction volume (see section

2.2.9). RestrictionMapper 3 (http://www.restrictionmapper.org/) was used in order to ensure that the fragment required for amplification was not digested by this restriction enzyme. Oligonucleotide primers for long PCR were designed with an in house programme (courtesy of David Beare); *longPCR*. Prior to primer design mouse repeat sequences from the region upon which the primers were based were repeat masked (RepeatMasker v.3.0.8). The designed primers were to amplify a region ~5-6 kb in length, upstream of the *Dgcr8* gene trap (see section 3.3.5 for a full description of primer design). An *AscI* site and a short, 4-base spacer were added to the 5' the end of each primer. The Expand 20 kb PLUS PCR System (Roche) was used for Long PCR with the MEXP62 PCR programme (see section 2.2.4). The PCR products were size separated by gel electrophoresis (see section 2.2.6) on a 0.7% agarose gel and purified with the Qiagen Qiaquick Gel Extraction kit. 2.3 µg of the fragment was then digested with the *AscI* restriction enzyme (NEB) in a 200 µl reaction (see section 2.2.9).

2.3.2 Preparing the pR3R4AsiSI plasmid

DB3.1 *E. coli* containing the pR3R4AsiSI plasmid (generously donated by the Skarnes laboratory) were streaked onto chloramphenicol and ampicillin selective LB agar plates and cultured overnight at 37˚C. A starter culture was set up in LB with ampicillin (8 hours, 37˚C, 300 rpm). This starter culture was then diluted 1/500 for overnight culture (37˚C, 300 rpm), again in LB with ampicillin. The plasmid was subsequently prepared by Qiagen HiSpeed Plasmid Maxikit and ethanol precipitated to increase the concentration. Then 5 µg of the plasmid was digested with an *AscI* (NEB) in a 200 µl reaction (see section 2.2.9). The digest was separated by 0.6% agarose gel electrophoresis (see section 2.2.6) and purified with a Qiagen Qiaquick

Gel Extraction kit. ~1 µg of the restricted plasmid was treated with Antarctic Phosphase (NEB) in a volume of 50.5 µl, according to the manufacturers instructions.

2.3.3 Cloning the *Dgcr8* fragment

The *Dgcr8* fragment was cloned into the prepared pR3R4AsiSI backbone using the Roche Rapid Ligation Kit according to the manufacturer's recommendations. The ligated plasmid was then transformed into MACH1 cells (Invitrogen) (see section 2.2.7.2). In order to judge which bacterial colonies contained the correctly inserted fragment, colony PCR (see section 2.2.5) was conducted with primers 13, 14, 15, 16, 17 and 18 (see section 2.2.4.1), paired as appropriate (See Fig.3.4). The plasmid was purified with a Qiagen Qiaprep spin miniprep kit. The plasmid was sequenced by the Sanger Institute Core Sequencing Facility with primers 14, 15, 17 and 18 (see section 2.2.4.1).

2.3.4 Preparing the targeted trap vector

The *Dgcr8* fragment was transferred from the pR3R4AsiSI plasmid to the pL3/L4_(+)_GT1T2hygroP2EGFP plasmid (Generously donated by the Skarnes laboratory) with Gateway L/R Clonase II (Invitrogen) according to the manufacturers protocol. The pL3/L4_(+)_GT1T2hygroP2EGFP plasmid was then transformed into MACH1 cells (Invitrogen) (see section 2.2.7.2). Colony PCR (see section 2.2.5) was used to identify colonies containing the correctly inserted fragment, with primers 14, 17, 15 and 18 (see section 2.2.4.1). Colonies were cultured in LB with kanamycin and frozen.

A starter culture was set up in LB with kanamycin (8 hours, 37˚C, 300 rpm). The starter culture was subsequently diluted 1/500 in fresh LB with kanamycin and cultured overnight. The plasmid was purified by HiSpeed Plasmid Maxi Kit (Qiagen) according to the manufacturers protocol, using 1.5x the usual amount of P1, P2 and P3 solutions to increase prep yield. The plasmid was phenol chloroform purified (see section 2.2.1) and resuspended in 200 µl $T_{0.1}E$.

50 µg of the plasmid was digested with *HindIII* (NEB) in a 500 µl reaction (No Sucrose/Cresol solution, 15 µl of enzyme introduced over 3 hours at 37˚C). 0.2 µl of each reaction was size separated on a 0.7% agarose gel by electrophoresis (see section 2.2.6) alongside non-digested plasmid to ensure the digest had proceeded to completion. Finally the restricted plasmid was ethanol precipitated (see section 2.2.2), but the precipitation itself preceded for 15 minutes on ice prior to centrifugation. In addition, following the initial 70% ethanol wash a further 500 µl of 70% ethanol was added to the plasmid. In a tissue culture hood the ethanol was removed and the plasmid was air dried for 25 minutes. The pellet was resuspended at room temperature, overnight, in DPBS (-$CaCl_2$ and $MgCl_2$) (Gibco).

2.4 ES cell culture

Cell lines were maintained in ES Cell culture medium at 37˚C, 7% CO_2 in gelatinized tissue culture treated plates and flasks (48, 24, 12 and 6 well plates (Falcon), T25 and T75 flasks (Corning)). Plates were gelatinized by adding 0.1% gelatin to the well prior to plating and aspirating away the excess. Medium was supplemented with selective agents appropriate to the cellular genotype (See below for specific culture conditions (see section 2.4.2)). Medium was replaced every day, unless otherwise

stated in the text. Cell images were taken with an Olympus IX51 microscope and Olympus DPSoft software at 10x relief contrast. Spinning of cells was conducted in an Eppendorf centrifuge 5702.

Well Size	Media (ml)
48	0.6
24	1.5
12	3
6	6
T25	10
T75	30

Table 2.2: Media quantities used to culture ES cells

2.4.1 Splitting cells

2.4.1.1 Method 1

Cells were washed once with DPBS (-$CaCl_2$ and $MgCl_2$) (Gibco). 1x Trypsin was then added to the well. Cells were incubated for 2 minutes at 37˚C. Trypsin was diluted 1:10 with ES cell culture medium. Cells were spun out of the medium (1,200 rpm, 3 minutes) to remove trypsin and the cell pellet was resuspended in fresh ES cell culture medium. The appropriate proportion of the cells was transferred to a fresh well or flask. The well/flask was topped up with an appropriate quantity of growth medium and the well/flask was mixed by pipetting.

2.4.1.2 Method 2

As Method 1, however, cells were incubated in trypsin for 3 minutes. In addition once the trypsin was diluted with fresh media the cells were transferred to fresh plates directly without spinning.

2.4.2 Growth conditions for specific cell lines

E14 mouse ES cells were used as a wild type control cell line (*Dgcr8*$^{+/+}$) as this is the parent cell line for BayGenomics gene trap cell lines used as the basis of this study. These cells were split every 2-3 days as they approached confluence. The normal split ratios were 1/8-1/10 and depended upon the condition and confluence of the cells.

Heterozygous gene trap cell lines from BayGenomics were generally maintained in media supplemented with 150 µg/ml G418 (Geneticin – Gibco). These cells were split every 2-3 days as they approached confluence. The normal split ratios were 1/8-1/10. These culture conditions were maintained upon the removal of selection prior to RNA lysis. Prior to electroporation these cells were split by Method 2 (see section 2.4.1.2). Due to a labeling problem in transit from the supplier (the labels fell off when thawing the vials) it is not possible to distinguish the XH157 and XG058 cell lines and be sure of which is which without a specific PCR or cloning strategy. This is because the gene traps in these cell lines are within the same intron. Hence from this point the cells have been arbitrarily ascribed the names *Dgcr8*$^{gt1/+}$ and *Dgcr8*$^{gt2/+}$. Subsequently these cell lines have been demonstrated to behave very similarly (Chapter 4 and 5) and as the identity of the cell lines appears to have limited biological relevance it was decided that these experiments would prove time consuming and of little significance.

The *Dgcr8*$^{tm1,gt1/+}$ and *Dgcr8*$^{tm1,gt2/+}$ cell lines were maintained in media supplemented with 100 µg/ml hygromycin B (Calbiochem). These cells were split every 2-3 days as they approached confluence. The normal split ratios were 1/3.5-1/5. Upon the removal of selection for experiments the split ratio varied between 1/3.5-1/6.

Dgcr8$^{gt1/tm1}$ and *Dgcr8*$^{gt2/tm1}$ cells lines were maintained in media supplemented with 100 µg/ml hygromycin B (Calbiochem). These cells were split every 2-3 days as they approached confluence. The normal split ratio was 1/3.5. Upon the removal of selection for experiments the split ratio varied between 1/3.5-1/6.

Dgcr8$^{gt/tm1}$ and *Dgcr8*$^{tm1,gt1/+}$ cells generally varied between passages 13 and 26 when used for experiments. The cells behaved consistently throughout.

2.4.3 Freezing ES cells

Cells were trypsinised as if to be split and the trypsination was stopped with a 1:10 dilution of ES cell culture medium (see section 2.4.1). The cells were spun out of this medium (1,200 rpm, 3 minutes) and resuspended in an appropriate volume of freeze medium (500 µl per vial; culture medium supplemented with 10% Dimethyl sulphoxide (DMSO) and filter sterilised). Cells were transferred to Nunc CryoTube vials and placed at -70˚C. Subsequently the cells were moved into liquid nitrogen storage.

2.4.4 Defrosting cells

In the evening, cells were removed from liquid nitrogen and warmed in the hand until defrosted. Cells were immediately transferred drop by drop to 10 ml of non-selective culture medium. Cells were swirled to mix and spun at 1,200 rpm for 3 minutes to pellet. The size of the pellet was used to judge the appropriate size of well into which the cells should be plated (See table 2.3). The pellet was subsequently resuspended in the appropriate quantity of non-selective culture medium and transferred to a

gelatinized plate. Early next morning the medium was replaced with the selective medium that corresponds to the cells' genotype.

Pellet size (mm) *Dgcr8*$^{+/+}$	Pellet size (mm) *Dgcr8*$^{gt/tm1}$	Well Size	Media for defrost
Barely visible	1mm	48	1ml
1mm	2mm	24	2.4ml
2mm	3mm	12	4ml
3mm	NA	6	8ml

Table 2.3: Culture plates and media quantity used for defrosting ES cells

2.4.5 Subcloning

Cells were trypsinised for 3 minutes. Trypsination was stopped by the addition of media. Cells were spun out of media and resuspended in fresh non-selective media. Cells were mixed gently to ensure they reached a single cell suspension. 10^4 cells were plated to 100 mm tissue culture treated plates (Corning) with 10 mls of 150 µg/ml G418 selective media (Geneticin – Gibco). Plates were fed every other day with 10-12 ml of fresh selective media. 10-11 days later 12 colonies of each cell line were picked (see section 2.4.6).

2.4.6 Colony picking

Culture medium was aspirated from the 100 mm (Corning) plate and replaced with 10 ml DPBS (-$CaCl_2$ and $MgCl_2$) (Gibco). Colonies were transferred with a pipette to a gelatinized 48 well plate in 50 µl DPBS (-$CaCl_2$ and $MgCl_2$). 50 µl 2x Trypsin was added to each well and incubated at 37˚C for 5 minutes. 1ml of 150 µg/ml G418 selective medium (Geneticin – Gibco) was added to the wells and they were mixed gently to disperse the cells. Subsequently cells were maintained under 150 µg/ml

G418 selective medium. Ultimately, 2 vials of each cell line were frozen (see section 2.4.3) and RNA was purified from each cell line by the Promega SV Total RNA Isolation System (see section 2.7.3) for RT-PCR to check the cell line genotype.

2.4.7 Gene targeting/electroporation

Cells were seeded from T25 to T75 and cultured for 2 days, until approaching confluence. The T75 was trypsinised for 4 minutes and subsequently the cells were collected in 10mls of media. Cells were spun down for 3 minutes at 1,200 rpm. Cells were resuspended in DPBS (-$CaCl_2$ and $MgCl_2$) (Gibco), counted and spun once more. Cells (normally 2-5x10^7) were resuspended in 700 µl of room temperature PBS. Cells were transferred to a microcentrifuge tube containing the DNA to be transfected (~50 µg assuming 100% recovery from plasmid digest), pipetted up and down once to mix and transferred to a .4mm electroporation cuvette (BioRad). Cells were immediately electroporated with a Gene Pulser II (800 V, 3.0 µF with an approximate time constant of 0.04 ms). Cells were left at room temperature for 20 minutes to recover, diluted in non-selective medium and plated onto 3x 100 mm gelatinized plates (Corning) (5 x 10^6, 2.5 x 10^6 and 1 x 10^6 cells). The following day, the media was replaced with 10 ml of media containing 120 µg/ml hygromycin B. The media was replaced daily for 2 weeks. After 9 days selection was dropped to 100 µg/ml hygromycin B to aid cell growth. From 11 to 15 days after plating, colonies of a suitable size were picked to 48 well plates (see section 2.4.6 substituting G418 selection for hygromycin selection). Subsequently cells were maintained in 100 µg/ml hygromycin B. RNA was purified from an over confluent 24-well-plate well of each cell line by SV RNA Purification (see section 2.7.3) for genotyping by RT-PCR. Ultimately 70%-90% confluent 48 well plates containing cultured colonies were

frozen by replacing the growth media with freeze media and storing them at -70˚C until genotyping was complete. To defrost, cells were removed from the -70˚C freezer and as soon as the freeze media melted it was replaced with >1ml of non-selective media. The next day this was replaced with selective, 100 µg/ml hygromycin B growth media.

2.5 Judging ES cell characteristics

2.5.1 Xgal staining

Cells were washed once with PBS and then Fix Buffer was added. Plates were incubated for 30 minutes at room temperature. Each well was then washed twice with wash buffer. Stain Buffer was then added to the cells and they were incubated overnight at 37˚C in a sealed Tupperware box. Subsequently Stain Buffer was removed from the cells, the cells were washed once with wash buffer and each well was stored under Fix Buffer. Cells were imaged at a magnification of 10x with relief contrast.

2.5.2 Immuno-staining

2.5.2.1 Preparing the slides

Prior to plating to slides, all cells were maintained for 2 days in non-selective media. On the second day cells were plated to gelatinized chamber slides (Nunc 8 well slides, VWR 62407-335). 2.1 x10^4 *Dgcr8*$^{tm1,gt1/+}$ cells and 4.2 x 10^4 *Dgcr8*$^{gt1/tm1}$ cells were plated in 300 µl of non-selective media per well. Approximately 24 hours later the media was removed and replaced with 300 µl of 4% paraformaldehyde solution. The slides were then incubated for 20 minutes at room temperature. The

paraformaldehyde solution was then replaced with 400 µl of DPBS (-$CaCl_2$ and $MgCl_2$) (Gibco) and the slides were incubated at room temperature for a further 5 minutes. Finally the DPBS was replaced with a further 400 µl of DPBS and the slides were wrapped in parafilm and stored at 4˚C.

2.5.2.2 Immuno-staining the slides

Each well was washed 3x with PBS. After the final wash, 100 µl of 5 mM NH_4Cl in PBS was added to each well and incubated for 10 minutes at room temperature. This solution was replaced with 0.2% Triton in PBS and incubated for 10 minutes at room temperature. The wells were washed a further 3x with PBS. Following the third wash the cells of each well were blocked with 100 µl of 5% donkey serum in PBS and incubated for 20 minutes at room temperature. Next the primary antibodies were added to the cells, diluted in 100 µl 5% donkey serum (Anti-Oct4 (Goat) Santa Cruz – sc8628 at 1/50 OR Anti-SOX2 (Rabbit) Chemicon International – AB5603 at 1/500). Slides were incubated for 1 hour at room temperature in a tip box with a damp cloth to maintain humidity. After an hour each well was washed 4x with PBS and blocked for a further 20 minutes with 5% donkey serum. Next the secondary antibodies were diluted in 5% donkey serum and 100 µl of the mixture was added to the appropriate wells for 1hr at room temperature (Alexa Fluor 594 Donkey anti-goat – Invitrogen – 1/500 OR Alexa Fluor 488 Donkey anti-rabbit – Invitrogen – 1/500). Subsequently cells were washed a further 4x with PBS. Finally the chambers were removed and the slides were dipped in RO water to rinse. A drop of Vectorshield with DAPI (Vector Laboratories) was added to each well. A cover slip was added and sealed to the slide with nail varnish. In parallel to the addition of primary and secondary antibodies to

the same wells, secondary antibodies were also applied to further wells with no primary antibody to allow an assessment of non-specific staining.

2.5.2.3 Imaging immuno-stained slides

A Leica TCS SP5 confocal microscope was used to take images of the slides at 20x magnification with oil. Oct4 images were taken with the following settings: Sequential setting 1 - Laser Line UV (405): 64%, PMT1 - ~412-489nm, Sequential setting 2 – Laser line Visible (594): 40%, PMT3 - ~608-690nm. SOX2 images were taken with the following settings: Sequential setting 1 - Laser Line UV (405): 100%, PMT1 - ~412-489nm, Sequential setting 2 – Laser line Visible (488): 75%, PMT2 - ~492-566nm. Laser power, Gain and Offset levels for each PMT were set to obtain the optimal image with minimum background. All settings were maintained for the images of both cell lines taken with relation to the same primary antibodies. A Z-series was taken of cells in each well (10 slices through 488 or 594 fluorescence visible in test wells and 5 slices through any 488 or 594 fluorescence visible in control wells). Final images were derived by a "maximum" projection of the Z-series and these were subsequently overlaid.

2.5.3 Optimised embryoid body (EB) formation protocol

Dgcr8$^{tm1,gt1/+}$ and *Dgcr8*$^{gt1/tm1}$ cells were cultured for 4 days in non-selective media prior to EB formation. A T25 flask of cells was trypsinised for 3 minutes. This reaction was stopped with the addition of media without LIF (-LIF). The cells were spun down (1,200 rpm for 3 minutes) and resuspended in 10 ml –LIF media. Cells were counted and diluted to 10^4 cells per ml for *Dgcr8*$^{+/+}$ and *Dgcr8*$^{tm1,gt1/+}$ lines and 4 x 10^4 cells for *Dgcr8*$^{gt1/tm1}$ cells. Cells were subsequently plated to a non-tissue culture

treated, round-bottomed, 96 well plate; 100µl per well. The following day a further 100 µl of -LIF media was added. On day 3, EBs were transferred to 24 well low attachment plates (Costar – 3473) and fed with 1.5 ml –LIF media. From day 4 onwards EBs were fed every other day. On days 5-8 half of the EBs were cultured with retinoic acid (RA) (1 µM). On day 8 the EBs were transferred to gelatinised tissue culture treated 12 well plates (Falcon). On day 12 the media was removed from the EBs and 0.8 ml 4% Paraformaldehyde Fixing Solution was added to the wells. EBs were fixed for 20 minutes at room temperature and then washed twice with PBS before being stored at 4˚C in PBS.

2.5.4 Cell cycle assay

2.5.4.1 Cell growth

Dgcr8$^{tm1,gt1/+}$ and *Dgcr8*$^{gt1/tm1}$ cells were cultured for 2 days in non-selective media prior to plating for cell cycle analysis. Cells were plated to gelatinised T75 flasks (Cell numbers: 186 x 10^4 *Dgcr8*$^{+/+}$ cells, 246 x 10^4 *Dgcr8*$^{tm1,gt/+}$ cells and 304 x 10^4 or 380 x 10^4 *Dgcr8*$^{gt1/tm1}$ cells) in non-selective media. After 2 days the cells were 60-75% confluent. Cells were washed with DPBS (-$CaCl_2$ and $MgCl_2$) (Gibco) and trypsinised for 4 minutes with 3ml 1x Trypsin. The reaction was stopped with 17 ml non-selective media and the cell suspension was spun for 3 minutes at 1,200 rpm. Cells were washed in 20 ml DPBS (-$CaCl_2$ and $MgCl_2$) (Gibco) and respun. The pellet was subsequently resuspended in 1 ml DPBS (-$CaCl_2$ and $MgCl_2$) (Gibco) and 9ml of ice cold 70% ethanol was added dropwise while vortexing. Cells were stored in this ethanol at 4˚C.

2.5.4.2 Fluorescence activated cell sorting (FACS)

Cell spinning for staining was conducted with a Thermo Electron Corp Heraeus Sepatech Megafuge 1.0. 5 mls of cell suspension was spun for 5 minutes at 1,200 rpm to pellet cells. Cells were washed once in 10 ml and once in 5 ml of PBS, spinning between washes to remove supernatant. Cells were then counted in the second wash and spun out of PBS. Cells were resuspended in 1 ml of propidium iodide (PI) solution per 2 x 10^6 cells. Cells were stained overnight and filtered to remove clumps (30μm mesh filter, Partec).

Stained samples were analysed with a Beckman Coulter (Cytomics FC-500) flow cytometer with a 20mW 488nm air-cooled argon laser. A 620nm band pass filter was used to collect the fluorescence emitted by the PI stained cells. 20,000 events were collected per sample.

2.5.4.3 FACS analysis

The cell cycle profile of each cell line was determined with *FlowJo* v7.2.5. Samples were gated to remove the signal from cell doublets and the Watson algorithm was used at default settings to determine the cellular profile.

2.6 Protein purification and Western blots

2.6.1 Protein purification

Trapped cells were maintained for 2 days in non-selective media. Subsequently all cells were plated to gelatinised tissue culture treated 100mm plates (Corning). 136 x 10^4 *Dgcr8*$^{+/+}$ cells, 180 x 10^4 *Dgcr8*$^{tm1,gt/+}$ cells and 223 x 10^4 *Dgcr8*$^{gt/tm1}$ were seeded per plate. Cells were maintained for a further 2 days in non-selective media and then

lysed at 70-85% confluence. Media was aspirated from cells and they were washed with DPBS (-$CaCl_2$ and $MgCl_2$) (Gibco) (2x20 ml). 360 µl of Protein Lysis Buffer was added to the cells and the plates were placed on ice for 5 minutes. The base of the well was scraped with a pipette tip and the lysate was transferred to an Eppendorf tube. The lysate was passed repeatedly (16x) through a 21G hypodermic needle and then spun at 14,000 rpm for 10 minutes at 4^{o}C. The lysate was then removed from the cellular debris and placed in a fresh tube. Lysate was snap frozen with dry ice and ethanol and stored at -70^{o}C. Samples were diluted 1/50 and the protein was quantified with Bradfords Reagent, according to the manufacturers protocol.

2.6.2 Western blot

50ug of each protein sample was separated on 4-12% Bis-Tris Gels (Invitrogen) using an XCell SureLock Mini-Cell (Invitrogen). Protein samples were prepared as described by the manufacturer with the inclusion of 1 µl of NuPAGE Reducing Agent. Gels were run in MOPS SDS Running Buffer (Invitrogen) in parallel with 3 µl of MagicMark XP protein standard (Invitrogen). Proteins were transferred to Hybond-ECL filter (Amersham) in a Mini-Trans-Blot Cell (BioRad) in Western Transfer Buffer (10% Methanol, 1x NuPAGE Transfer Buffer (Invitrogen)) at 100 V for 1.25 hours.

Filters were blocked for 1 hr in Western Blocking Solution with gentle rocking. Subsequently the blocking solution was replaced with fresh solution and the primary antibodies were added. The anti-Oct4 (1/1000 Santa Cruz – sc8628) and the anti-α-tubulin antibody (1/5000 Abcam – ab7291) were added together. The filters were rocked gently for 1 hour. Each membrane was rinsed 3 times for 10 minutes in

Western Washing Solution with gentle rocking. Initially the peroxidase conjugated secondary antibody appropriate for the Oct4 primary antibody (1/7500 Sigma-Aldrich A4174) was added in Western Blocking Solution and the filters were rocked for 2 hours. The Blocking Solution was subsequently removed and the filters were rinsed 3x in Western Washing Solution for 10 minutes with rocking. 5mls of Western Lightning Enhanced Luminol Reagent and 5 ml Western Lightning Oxidising Reagent (Perkin Elmer) were added to the membranes and they were incubated for 1 minute in the dark with rocking. Excess fluid was blotted from the membranes with 3MM Whatman paper and the membranes were wrapped in Saran wrap and exposed to film for a variety of durations to optimize the image obtained. The filters were then washed 3 times with Western Washing Solution for 10 minutes with rocking and then Western Blocking Solution was added with a 1/7500 dilution of peroxidase conjugated secondary antibody (Sigma-Aldrich A6782, appropriate for binding the anti-α-tubulin primary antibody). Membranes were incubated for a further hour with this solution, rinsed 3 times and developed as described above.

2.7 RNA purification

2.7.1 Trizol

Cells were lysed with Trizol reagent (Invitrogen). Cells were washed twice with DPBS (-$CaCl_2$ and $MgCl_2$) (Gibco) and Trizol was added (7.5 ml per T75, 1 ml per 6 well). The plate was rocked gently. After the Trizol cleared it was homogenized by pipetting and transferred to a Falcon tube. Lysate was stored at -70˚C. RNA purification essentially followed manufacturers recommendation. However, large volumes of Trizol chloroform mix (T75) were centrifuged at 4200 rpm (Beckman J6-MC) for 1 hour at 4˚C in a 50 ml Falcon tube to separate the aqueous phase. The

aqueous phase was subsequently divided between 5 Eppendorfs tubes for ethanol precipitation of the RNA. Smaller volumes of Trizol:chloroform (6 well) were phase separated with Eppendorf phaselock gel tubes (Heavy Gel) spun for 15 minutes at < 12,000g, 4˚C. RNA pellets were stored under 75% ethanol at -20˚C. Pellets were resuspended in RNase free water for applications. Once resuspended RNA was stored at -70˚C. RNA was generally quantified by Nanodrop (Although early quantification was performed by Eppendorf Bio photometer) and an aliquot was size separated by agarose gel electrophoresis (see section 2.2.6) to ensure its integrity before use.

2.7.2 DNase treatment

When mentioned in the text Trizol purified RNA was TURBO DNase treated according to the manufacturers protocol (Ambion). DNase treated RNA was cleaned with a Qiagen RNeasy MiniElute Cleanup Kit according to the manufacturers protocol.

2.7.3 SV purification

RNA was purified using the SV Total RNA isolation System (Promega) from a single, confluent 24-well-microtitre-plate well of ES cells. Cells were lysed with 300 µl SV RNA Lysis buffer and incubated at 70ºC for 3 minutes, followed by centrifugation at 12,000-14,000 g at room temperature for 10 minutes. 200 µl of 96% ethanol was added to the cleared lysate. The lysate was passed through a SV Total RNA Isolation System spin column. From this point on the manufacturers recommendations were followed. RNA was quantified by Eppendorf Bio photometer.

2.7.4 Harvesting HeLa S3 RNA

HeLa S3 cells were cultured in suspension in Hams F12 media supplemented with 10% Foetal Bovine Serum and 1x Penicillin-Streptomycin-Glutamine (Invitrogen). 3x T75 flasks containing a total of approximately $5.6x10^7$ cells in 180 ml media were lysed for RNA with Trizol. Briefly, cells were separated into 6 50 ml falcons and spun at 1,200 rpm for 5 minutes to pellet. Each pellet was resuspended in 5 ml PBS, combined to a single 50 ml Falcon and spun again to pellet. The pellet was resuspended in 30 ml PBS to wash, spun and lysed with 7.5 ml Trizol. Lysate was stored at -70˚C. Lysate was processed as explained in section 2.7.1.

2.8 RT-PCR

Roughly 300 ng of SV purified RNA (or 100ng of DNase treated Trizol purified RNA if stated in the text) was reverse transcribed with Superscript II (Invitrogen) according to the manufacturers instructions. Reverse transcription was primed by either random hexamer (Roche) or oligo dT primers (Invitrogen).

Nested PCR was conducted to amplify fragments from the complementary DNA (cDNA). This consisted of 2 rounds of 15 µl or 25 µl KOD Hot-Start PCR reactions (see section 2.2.4). The template for the first round was 1-2 µl of the previously described RT reaction primed by the external primer set. The second round template was 1-2 µl of a 1/100 to 1/500 dilution of the completed first round reaction and was primed by an internal primer set. The MKOD6030 PCR programme was used for each round (see section 2.2.4.2). 3 µl of the product of the second reaction was size separated by agarose gel electrophoresis (see section 2.2.6).

For sequencing the products of the complete second round reaction were size separated by agarose gel electrophoresis and the bands of the correct size were excised from the gel and purified using the Qiagen Qiaquick Gel Extraction Kit according to the manufacturers instructions. 4µl of the purified fragments were subjected to gel electrophoresis to check the specificity of the band purification (see section 2.2.6). Purified bands were sequenced by the Sanger Core Sequencing Facility (see section 2.2.8).

2.9 mRNA expression profiling

2.9.1 Northern blot

2.9.1.1 Northern blot preparation

Dgcr8$^{tm1,gt1/+}$, *Dgcr8*$^{tm1,gt2/+}$, *Dgcr8*$^{gt1/tm1}$, *Dgcr8*$^{gt2/tm1}$, *Dgcr8*$^{gt1/+}$ and *Dgcr8*$^{gt2/+}$ cell lines were cultured for 2 days in non-selective media and then all cells were plated to T75 flasks (Cell numbers: 186 x 10^4 *Dgcr8*$^{+/+}$, *Dgcr8*$^{gt1/+}$ and *Dgcr8*$^{gt2/+}$ cells, 246 x 10^4 *Dgcr8*$^{tm1,gt1/+}$ and *Dgcr8*$^{tm1,gt2/+}$ cells and 304 x 10^4 *Dgcr8*$^{gt1/tm1}$ and *Dgcr8*$^{gt2/tm1}$ cells). RNA was purified by Trizol (see section 2.7.1) and 100 µg of RNA was cleaned with an RNeasy Mini Kit according to the manufacturers protocol. The RLT and RPE washes were collected as these contain the small RNA fraction, suitable for Solexa sequencing. RNA was eluted in 50 µl of water. The eluate was subsequently reapplied to the same column and re-eluted to maximise RNA yield. The RNA in the eluate was quantitated with an Agilent Technologies 2100 Bioanalyser using a EukaryoticTotal RNA Nano Chip. Half of the eluate was then processed with the PolyATract mRNA Isolation System III (Promega) to remove non-polyadenylated RNA, including ribosomal RNA (rRNA), from the samples. The concentration of the polyadenylation (polyA) selected RNA was quantified again by Bioanalyser, as

above, to ensure the removal of rRNA was successful. All subsequent centrifugation steps were conducted with a Thermo Electron Corp Heraeus Pico 21 centrifuge at maximum unless otherwise stated. The mRNA was ethanol precipitated (625 µl 100% ethanol, 25 µl 3 M Sodium Acetate, 3 µl GlycoBlue (Ambion); overnight at -20˚C; Spin 60 minutes, 4˚C, 700 µl 70% ethanol wash; resuspend in 10 µl RNase free water). The original, pre-polyA selection Bioanalyser results were used to estimate approximately equal quantities to load on the Northern and the samples were made up to 10 µl with RNase free water. The RNA was mixed with 2x RNA loading buffer (17% formaldehyde, 50% formamide, 1x MOPS, 5% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol), heated to 70˚C for 10 minutes and cooled on ice. A gel was prepared (1% Agarose, 1x MOPS, 2% Formaldehyde, 1/50,000 SybrGreen (Invitrogen)) and pre-run for 30 minutes at 70 V, 4˚C in 1x MOPS Buffer. The samples were loaded alongside a 0.5-10 kb ladder (Invitrogen). Samples were run for ~5 hours at 70-200 V, 4˚C. Nucleic acids were visualised with UV light on a transilluminator and digital images were captured with a UVP system. The gel was rinsed in 10x SSC. The gel was transferred over night by capillary blot to a Hybond XL membrane (GE Healthcare) with 10x SSC and UV cross-linked (UVP).

2.9.1.2 Northern probes

Primers were designed using ENSMUST00000115633 and ENSMUST00000009321 as the template sequences. *Dgcr8*$^{+/+}$ Trizol-purified-RNA derived cDNA was used as the template for probe amplification. Probes were amplified in 25 µl KOD Hot-Start PCR (Novagen) reactions (see section 2.2.4) using primers 25 and 26 (3') or 27 and 28 (5') (see section 2.2.4.1), following the MKOD6030 programme. Amplified probes were purified by gel electrophoresis (see section 2.2.6) followed by Qiagen Qiaquick

Gel Extraction. The fragments were A-tailed (1 µl NEB Buffer, 2 µl dATP (Amersham), 1 µl AmpliTaq (Perkin Elmer), ~250ng DNA (3-6 µl), to 10 µl with DDW. 70˚C for 30 minutes), ligated into the pGEM-T-Easy vector (Promega) with the Roche Rapid Ligation Kit and transformed into MACH1 cells (Invitrogen) (see section 2.2.7.2). The successful transformant colonies were Blue/white selected on Xgal containing ampicillin selective plates (Cultured overnight at 37˚C). Colony PCR was conducted with flanking plasmid primers 29 and 30 to check the fragment insertion (see section 2.2.6). Plasmids containing the correct inserts were prepared with a Qiaprep Spin Miniprep Kit (Qiagen). These plasmids were sequenced with primers 25, 26, 27, 28, 29 and 30 as applicable (see section 2.2.8). The probes were finally amplified by KOD Hot-Start PCR in 25 µl reactions, with a 1/10,000 dilution of the correctly identified, purified plasmids as template and probe specific primers given above. The total PCR reaction was subjected to gel electrophoresis and the probes were purified by Qiaquick Gel Extraction (Qiagen).

2.9.1.3 Hybridisation

Probe labeling and hybridization were performed by Dr. Claudia Kutter. Probes were radiolabeled with [a32P]-dCTPs using the Random Labeling kit (Invitrogen). The probe was subsequently purified with a G50 spin column. Pre-hybridisation and hybridisations were performed using 15 ml PerfectHybTMPlus buffer (Sigma) at 55˚C in a rolling incubator. The 5' probe was added to the hybridization buffer and hybridized overnight at 55˚C. Filters were washed once with Wash Buffer 1 (2x SSC, 0.1% SDS) for 5 minutes at room temperature. The filters were washed a further 2x in Wash Buffer 2 (0.1x SSC, 0.1% SDS) at 55˚C for 10 minutes and 30 minutes respectively. The filters were wrapped and exposed to a phosphoimager screen (GE

Healthcare) for 2.5 hours. The filter was subsequently stripped twice with boiling 0.1% SDS for 20 minutes until only background radioactivity was detected. The 3' probe was hybridized as before and exposed to a phosphoimager overnight.

2.9.2 Illumina expression arrays

2.9.2.1 Expression array preparation

For standard expression array profiling, unless otherwise stated, *Dgcr8*$^{tm1,gt/+}$ and *Dgcr8*$^{tm1,gt/+}$ cells were cultured for 2 days in non-selective media and then all cells were plated to T75 flasks (Cell numbers: 186 x 10^4 *Dgcr8*$^{+/+}$ cells, 246 x 10^4 *Dgcr8*$^{tm1,gt/+}$ cells and 304 x 10^4 *Dgcr8*$^{gt1/tm1}$ cells). Following 2 days of further culture sub-confluent cells were lysed with Trizol (see section 2.7.1). For expression profiling post miRNA mimic reintroduction cells were cultured in 6 well plates as described in the miRNA transfection section (see section 2.11.2.2).

10µg of Trizol purified RNA was cleaned up with an RNeasy MiniElute Cleanup Kit (Qiagen) according to the manufacturers protocol. The RNA was then quantified by Nanodrop and 500 ng of RNA was amplified and labeled with the Illumina Total Prep RNA Amplification Kit (Ambion) according to the manufacturers protocol either by me or by the Sanger Institute Microarray Facility.

2.9.2.2 Expression arrays

Microarrays were processed by the Sanger Institute Microarray Facility. Briefly, 1500 ng of biotinylated cRNA was hybridised to Illumina Mouse-6 v1.1 Expression BeadChips overnight at 58˚C. These chips were washed, detected and scanned,

following the manufacturer's instructions. The scanner output was imported into BeadStudio software v.3.1.8 (Illumina).

2.9.3 Computational analysis of expression arrays

Array analysis was conducted with advice and help from Dr. Cei Abreu-Goodger. The general analysis of the expression array data was conducted in R 2.8.1/Bioconductor (http://www.bioconductor.org/) with additional packages (*affy*, *lumi*, *limma*, *ape*, *lumiMouseAll.db*, *GOstats*, *GO.db*, *R2HTML*, *annotate*, *KEGG.db* and *org.Mm.eg.db*). The function *addNuID2lumi* was used to ascribe nuID information to probe IDs, via probe sequences, from the Illumina Mouse-6 v1.1 annotation file *Mouse-6_v1_1_sequence.csv*. All arrays for both cell line expression analyses and miRNA transfection experiments were VST transformed (Lin et al., 2008b) and quantile normalised together.

2.9.4 Relationship plots

Sample relationships were determined using a distance metric based on Spearman rank-correlations; (1-cor). A distance tree was then computed using the Saitou and Nei (1987) method. Unrooted trees were plotted using the *ape* package. The subset of probes selected for correlation calculations was refined using the detection call from the Illumina Beadstudio output, restricting probe choice to only those called as "present" with a *P*-value < 0.05 in more than five of the samples. For further refinement, the normalised expression arrays corresponding to the cell line profiling arrays grown with culture method 2 (Section 5.3.1) were considered independently from the other array sets. A linear model was constructed including the arrays from all 5 cell lines (*Dgcr8*$^{tm1,gt1/+}$, *Dgcr8*$^{tm1,gt2/+}$, *Dgcr8*$^{gt1/tm1}$, *Dgcr8*$^{gt2/tm1}$ and *Dgcr8*$^{+/+}$). The

limma package was used to determine the genes with significant change in expression when comparing the *Dgcr8*$^{gt1/tm1}$ and *Dgcr8*$^{gt2/tm1}$ cells to the *Dgcr8*$^{tm1,gt1/+}$ and *Dgcr8*$^{tm1,gt2/+}$ cell lines (*P*-value = 0.05, Log fold change = $\log_2(1.2)$). Subsequently "detected" and "significant" probes were used to recalculate the correlations and plot the relationships between all samples.

2.9.5 Identification of expression changes between cell lines

In order to compile gene lists for Sylamer analysis, four sets of comparisons were performed. First of all the normalised expression arrays from each growth method (see section 5.3.1) were considered separately using the *limma* package to generate probe lists ordered by log fold change and t-statistic as a result of comparisons between cells of differing genotypes. Similar gene lists were compiled using *limma* when all cellular expression arrays were considered together irrespective of the culture conditions both in the analysis and in the construction of the linear model upon which the analysis is based. In this case the method by which the cells were grown was provided to the linear model as an additional factor. The miRNA transfection experiment arrays were excluded from these analyses. The final comparisons were made between the post miRNA transfection expression arrays. The linear model for this comparison was constructed using the normalised array data from only the miRNA transfection experiments. Sylamer analyses (van Dongen et al., 2008) were performed by Dr. Cei Abreu-Goodger (seeds were defined as bases 7mer-1A and 7mer-m8. 740 mouse miRNA 7mers were compiled from miRBase version 12. *P*-values were Markov corrected based on words of length 4). In addition to ordered gene lists *limma* was also used to identify significant expression changes

between samples using the linear models constructed above, see text for the details of these comparisons.

2.9.6 KEGG and GO analysis

For gene ontology (GO) analysis probes with an inter-sample interquartile range (IQR) less than the median IQR for all probes were removed from the analysis. Next probes without Entrez IDs annotated in the *lumiMouseAll* library were also removed along with probes without a *lumiMouseAll* annotated GO term. Only a singe probe, with the greatest IQR, was used for the analysis from sets of probes which target the same gene. Finally probe IDs were converted into Entrez IDs using the *lumiMouseAll* library. This gene set was used as the "gene universe" for the comparison. Lists of probes with significantly altered expression were filtered in the same way. Finally the *GOstats* package was used to calculate a *conditional* hypergeometric P-value for GO term enrichment (The *conditional* parameter calls on the algorithm to estimate whether a term is significantly enriched beyond the evidence contributed by a terms' child terms within the GO tree). A *P*-value cut off of 0.01 was applied for the analysis. The categories were then filtered to leave only those containing 10 or more gene IDs. A similar process was applied for Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. However, probes were filtered for those with annotated KEGG terms rather than GO terms and the Category size was not limited. In addition the KEGG terms for the analysis were derived from the *org.Mm.eg.db* library.

2.9.7 Wilcoxon Rank Test of the expression changes of the targets of transcription factors (TFs) when compared to the general expression changes in DGCR8 depleted cell lines

The TF target predictions were downloaded from the supplementary material of Kim *et al.* and Chen *et al.* (Chen et al., 2008;Kim et al., 2008b). Targets proposed by Chen *et al.* with an "association score" greater than 0 were considered as potential TF targets. For each TF these targets were intersected with those of Kim *et al.* and targets predicted by both sets were used. The Illumina array probes were ordered according to log fold change (LFC) in a comparison between $Dgcr8^{tm1,gt/+}$ and $Dgcr8^{gt/tm1}$ cell lines. Probes were converted to gene symbols with the *lumiMouseAll* library. Probes with no symbol annotation were removed. For multiple probes annotated to the same gene, the mean LFC was used and the duplicate was removed. Genes present in the TF lists but not present on the array were removed. For each TF their targets' associated LFC values were compared to the LFC values of the remainder of the genes on the array with a Wilcoxon Rank Sum test.

2.9.8 Annotation of probes associated with potential miRNA targets

The log fold change and *P*-value scores in the relevant microarray experiments were included (Sections 5.3.2.1 and 5.3.3.5). Gene symbol, gene name and Entrez ID were annotated using the *lumiMouseAll* annotation library. In order to build a more comprehensive annotation file, if no associated Entrez ID or gene name was available in this library an attempt was made to fill these gaps with an annotation file constructed by Dr. Cei Abreu-Goodger, directly from the Ensembl database (release 51). This file was also used as a source of Ensembl annotated transcripts mapped to each probe to be used for Sylamer seed counts. Vega transcripts

(http://vega.sanger.ac.uk/index.html) were selected in preference, if these were not present Ensembl transcripts (http://www.ensembl.org/index.html) were used and, if these were unavailable, EST (Expressed Sequence Tag) based transcripts were selected. If multiple transcripts were available within the same class, the transcript with the longest 3'UTR was used. The Sylamer programme was used to screen these UTRs for miRNA associated seed sequences (Dr. Stijn van Dongen) (van Dongen et al., 2008). Probe associated GO terms were identified with the *lumiMouseAll* database. These were filtered for GO terms from the "Biological process" ontology using the *annotate* package and these IDs were included in the probe annotation table. GO descriptions associated with these IDs were derived from the *GO.db* library. Probe associated KEGG pathway IDs were again derived from the *lumiMouseAll* library and the associated pathway names obtained from the *KEGG.db*. TargetScan version 4.2 (Grimson et al., 2007;Lewis et al., 2005) predictions were identified through the EntrezIDs associated with each probe. To do this mouse EntrezIDs were mapped to Human EntrezIDs using NCBI homologene annotation (http://www.ncbi.nlm.nih.gov/homologene). If multiple TargetScan predictions for the same gene were available (http://www.targetscan.org/vert_42/), the one with the best context score was recorded. miRBase v5 targets (http://microrna.sanger.ac.uk/targets/v5/) were also queried using the probe associated Entrez ID, where available. These were mapped to Ensembl transcripts using miRBase annotation, by Dr. Stijn van Dongen and subsequently used to identify transcript-associated targets and their "P-orthologous group" values. The smallest "P-orthologous group" value was included in the case of multiple predictions for the same gene. Finally, where multiple probes were associated with the same gene, the annotation was amalgamated to a single entry. In these cases the minimum *P*-values

and maximum log fold changes for associated array expression changes were included.

2.10 miRNA expression profiling

2.10.1 miRNA Northern blots

2.10.1.1 Preparing the blots

Unless otherwise stated cells were grown and RNA was purified as described in the section describing expression array preparation (see section 2.9.2.1). RNA samples were size separated by gel electrophoresis using 15% TBE-Urea gels (Invitrogen), in a XCell *SureLock* Mini-Cell, with Novex TBE-Urea Sample Buffer and Novex TBE Running Buffer (Invitrogen) according to the manufacturers protocol. 12.7 µg to 20 µg of total RNA was loaded per well. The same quantity of RNA was loaded in each well of a gel unless otherwise stated. RNA was run in parallel with a DNA oligo ladder (see Table 2.4). All buffers were prepared with HPLC grade water (BDH). Bottles and cylinders were rinsed with 0.1% SDS and 100 mM NaOH before use. Post-electrophoresis, the ladder was sliced from the gel and post-stained with ethidium bromide (Approximately 7 µl of ethidium bromide in 50 ml TBE) and visualised on a UV transilluminator and images were recorded (UVP).

The RNA was transferred to a Genescreen Plus nylon membrane (Perkin Elmer) using a BioRad Semi-Dry Electrophoretic Transfer cell. Briefly, the gel was equilibrated for 20 minutes in 0.5x TBE. 4 extra thick blot paper pads (BioRad) and the transfer membrane were soaked in 0.5x TBE and then stacked with the gel in the order of 2 pads, membrane, gel and then a further 2 pads. RNA was transferred at approximately

25 V for 45 minutes. While moist the membrane was UV crosslinked with a stratalinker (Stratagene) with 1000 µJ total energy and baked for 1 hour at 80˚C.

Ladder	Oligo Sequence
97nt	TGCTGTTGACAGTGAGCGCTGCCTTGATGTTATTCCAGAG TAGTGAAGCCACAGATGTACTCTGGAATAACATCAAGGCA TTGCCTACTGCCTCGGA
65nt	GGCCAAGCTTGCCACCATGGACTACAAAGACGATGACGA CAAGGATATCTTAAGCGGCCGCGGCC
37nt	AATTGGCGCGCCGAAGTGTTTGGGAGATGTGCAGAGT
25nt	GTTAGAAGATTACCAAGATGCAGTG
20nt	ACATCTGCCAATCCATCTCA
18nt	GTTTTCGGGACCTGGGAC

Table 2.4: The sequences of DNA oligos used to make a size ladder for small RNA gel electrophoresis

2.10.1.2 Probe labelling

The probes used were DNA oligos complementary to the miRBase annotated miRNA targets (see Table 2.5). 2 µl of 10 µM oligo was added to 2 µl 10x T4 PNK kinase buffer (NEB), 2.5µl of ^{32}P-γ-ATP (Amersham AA0018), 12µl DDW and 1µl T4 PNK Kinase (NEB). The probe was incubated for 1 hour at 37˚C and heat inactivated for 10 minutes at 68˚C. The probe was purified with a G-25 MicroSpin column (GE Healthcare) according to the manufacturer's protocol.

2.10.1.3 miRNA Northern blot probes

Probe	Sequence	Published Source?
miR-16	CGCCAATATTTACGTGCTGCTA	
miR-21	TCAACATCAGTCTGATAAGCTA	
miR-92a	CAGGCCGGGACAAGTGCAATA	
miR-130a	ATGCCCTTTTAACATTGCACTG	
miR-292-3p	ACACTCAAAACCTGGCGGCACTT	Murchison *et al.* 2005
miR-293	ACACTACAAACTCTGCGGCACT	Murchison *et al.* 2005
miR-320	TTCGCCCTCTCAACCCTGCTTTT	
Let-7a	AACTATACAACCTACTACCTCA	
U6snRNA	GCTTCACGAATTTGCGTGTCATCCT	Murchison *et al.* 2005

Table 2.5: miRNA Northern blot probe sequences

2.10.1.4 Hybridisation of miRNA Northern blots

50ml Pre-hyb solution was preheated to 50˚C. Pre-hyb solution was then applied to the miRNA filter in a sandwich box and incubated for 2+ hours at 50˚C. Subsequently the Prehyb solution was replaced with 50 mls of fresh solution to which the prepared probe was added. The filter was incubated at 50˚C overnight and then washed twice with 40 ml Primary Wash Solution for 10 minutes. The filters were subsequently washed a further two times with primary wash solution with 20 µl of 100 mM ATP, added for 30 minutes. Finally the filters were washed for 5 minutes in 80 ml Secondary Wash Solution. In general the wash volumes were doubled if multiple filters were washed together. All washes were conducted at 50˚C with shaking. Finally the filters were sealed in saran wrap and exposed to film, with or without intensifiers, for varying periods to obtain the optimal image.

To strip filters, where necessary, boiling Strip Solution was poured on the filters and they were allowed to cool for 45 minutes to 1 hour with gentle rocking. This process was repeated if the first attempt did not remove sufficient probe. The filters were then rinsed in 2x SSC, wrapped in Saran wrap and exposed to film to judge the success of the stripping.

2.10.2 Luminex 100 analysis of mRNA expression

2.10.2.1 Running the Luminex 100 system

Cells were grown and RNA was purified as described in the section Expression Array Preparation (see section 2.9.2.1). RNA was quantitated by Nanodrop and the small RNA was labeled and bead coupled essentially as described in Blenkiron *et al.* (Blenkiron et al., 2007) with a few minor alterations. Briefly, 10 µg of each sample

was spiked with 3x precontrol oligos at 3 fmoles/sample and the 18-26 mer fraction was purified by PAGE using the BioRad Criterion System. 3' and 5' adapter RNA-DNA hybrid oligos (3': pUUUaaccgcgaattccagt-idT, 5': acggaattcctcactAAA) were ligated to either end of the size selected RNA fragments with T4 RNA ligase, in between successive rounds of PAGE size selection to isolate the successfully ligated fractions. The cloned RNA species were reverse transcribed with SuperscriptII and the M37 primer (TACTGGAATTCGCGGTTA) and the 8 µl of the cDNA sample was subsequently used as the template for PCR amplification of the library with primers M37 and M33 (5' Biotin-CAACGGAATTCCTCACTAAA). The PCR products were precipitated and resuspended in 64 µl TE pH8 (Sigma). 3 biotinylated post controls were spiked into the samples (100 fmols of each). A mouse miRNA specific set of oligo probes corresponding to 319 miRNAs and assorted controls, each coupled to a colour-coded polystyrene bead were used to profile the miRNA profile of each cloned sample. These probe were divided into four separate bead sets to allow each probe to be coupled to a specific bead colour. The control probes were included in each set. Each sample was hybridized to all 4 bead sets. 33µl of the prepared bead pools were combined with 16 µl of each sample and samples were incubated overnight at 50˚C. 1-2 bead blanks and negative TE pH8 (Sigma) controls were included with each sample. The beads were washed as described (Blenkiron et al., 2007) and a 1/100 dilution of SAPE (streptavidin R-phycoerythrin) was added to the beads. These were further incubated at 50˚C for 10 minutes to allow the Biotin to bind the SAPE. Finally the median fluorescence intensity (MFI) of each coloured bead in each bead set was determined with a Luminex 100 machine and Starstation software (ACS, Sheffield, UK).

2.10.2.2 Computational analysis of the Luminex data

The analysis of the Luminex data was conducted in R 2.8.1/Bioconductor (http://www.bioconductor.org/). The MFI values for each sample were $\log_2$ transformed. The first replicate samples were considered independently from replicates 2 and 3. The probes for each sample were divided into separate bead sets. The MFIs of samples within each bead set were normalised by using a factor equal to the mean of the MFIs for the precontrol beads for the sample divided by the mean of the precontrol MFIs in all samples, for the relevant bead set. By normalising each bead set separately no assumption was made as to the quality of signal from each of the beadsets. However, following a comparison of the corrected precontrol means for each bead sets I was confident that all of the bead sets were behaving in a similar manner. Subsequently I normalised all samples across all beadsets using factors determined by dividing the average of the precontrol MFIs within each beadset by the average of the precontrol MFIs in all beadsets.

2.10.3 miRNA expression profiling using Illumina/Solexa high-throughput sequencing

2.10.3.1 Preparing Illumina/Solexa samples

The RLT and RPE washes, from the RNeasy Mini Kit (Qiagen) purification of RNA for Northern blot (see section 2.9.1), were subjected to isopropanol precipitation to collect the small RNA fraction which they contain (see section 2.2.3). An Agilent Technologies 2100 Bioanalyzer was used to assess the concentration and integrity of the RNA with a small RNA chip (Agilent). The Solexa libraries were prepared according to the Illumina "Preparing Samples for Analysis of Small RNA" protocol version 1 (2007) with a few exceptions. The initial purification stage was skipped and

the process begun with the addition of the 3' adaptor to the small RNA fraction. The RNA was denatured at 92˚C for 2 minutes and snap cooled on ice. Subsequently a slightly altered reaction mixture was compiled (7.4 µl (240-560 ng) RNA, 1.1 µl SRA 3'Adapter, 3 µl DMSO (100%) (Qiagen), 1.5 µl RNA ligase Buffer, 1 µl RNase Out (Invitrogen), 1 µl RNA ligase). This reaction mixture was treated as described by Illumina and size separated on a Novex 15% TBE-urea gel, as described, with the SRA ladder and 10 bp DNA ladder (Invitrogen). The RNA between 35-65 bp was sliced from the gel and purified as described. The RNA was eluted from the gel slice overnight at 4˚C prior to the ethanol precipitation (which included 3 µl of GlycoBlue (Ambion) to aid pellet recovery). Precipitated RNA was resuspended in 3.9 µl of RNase free water, heat denatured as above, and the 5'Adapter was ligated with a slightly altered reaction mix (3.9 µl RNA, 1.1 µl SRA 5'Adapter, 2 µl DMSO, 1 µl RNA ligase Buffer, 1 µl RNase OUT and 1 µl RNA ligase). The reaction mixture was treated as described in the protocol and size separated on a 10% TBE-urea gel with both ladders as before. A band of 60-100 bp was sliced from the gel and eluted as before. The eluted RNA was ethanol precipitated and resuspended in 4.5 µl of ultrapure water. The RNA was reverse transcribed and the cDNA was PCR amplified in 4 separate reactions for each template (1 µl RT reaction, 4 µl HF Buffer, 0.125 µl RT primer (CAAGCAGAAGACGGCATACGA), 0.125 µl smRNA primer (AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA), 0.2 µl Phusion Taq (NEB), 12.55 µl Water). The PCR programme was as described by Illumina. A 10% SequaGel PAGE gel was prepared to allow space for maximum RNA separation. The Sequagels were pre-run for 30 minutes at 150 V. 6x Loading Buffer was added to the PCR reactions. These were then heat denatured at 65˚C for 15 minutes, snap chilled and the 4 PCR reactions for each sample were combined and

run in 2 wells of the gel alongside the 25 bp DNA ladder. The smRNA libraries were identified by size and sliced from the gel as described. The DNA was eluted from the gel slurry in 400 µl of 0.3M NaCl overnight at room temperature and then ethanol precipitated as before. Finally the libraries were resuspended in 10 µl Resuspension Buffer (Illumina). The concentration and integrity of the libraries were once again judged with an Agilent Technologies 2100 Bioanalyzer and a DNA 1000 Chip (Agilent). Libraries were stored at -80˚C. The Illumina libraries were Solexa sequenced by the Sanger Institute Core Sequencing Facility (36-cycle Single-ended run, Illumina GA instrument).

2.10.3.2 Computational analysis of the high-throughput sequencing data

The initial quality control and data analysis of the high-throughput sequencing data was conducted by Dr. Cei Abreu-Goodger. Briefly, 3' adapter sequence was removed from the reads. Reads that comprised of a single nucleotide for >75% of their length or less than 16 bp long following the removal of adapter were stripped from the dataset. An "RNA" database was constructed including all of the mouse miRBase miRNA hairpins and all of the mouse RNA genes from Ensembl (excluding the miRNA genes). Ssaha2 was used to match all unique sequence reads to this database (Ning et al., 2001). If a read matched to different kinds of RNA within this dataset, it was removed. The parameters chosen for ssaha2 required that the aligned region must be 100% identical, have a length of at least 16 bases and comprise >75% of the actual read length. The aligned region had to start at least at position 2 of the read and with no insertions/deletions (indels) in the aligned region. Alignments on the reverse strand were ignored and only the best scoring alignment(s) for each read was considered.

The accumulated read depth at each base position within all of the RNA genes was calculated. Mature miRNAs were considered present if at least half of their length was covered by a read depth ≥1, otherwise the miRNA was considered to possess a read depth of 0. For other miRNAs, the maximum read depth of the mature miRNA was used. For other RNA genes the maximum depth across the entire gene was considered. In addition to mapping the sequence reads generated as part of this study, the Illumina/Solexa data from the study by Babiarz *et al.* (GEO Database, GPL7195) (Babiarz et al., 2008) were also remapped against the same RNA database for comparison.

I conducted further analysis of the data in R 2.8.1/Bioconductor (http://www.bioconductor.org/) using packages *lattice*, *ape*, *affy*, *limma* and *gplots*. In order to normalise the maximum read depths between samples, first RNAs with a maximum depth of 0 in all samples were removed from the dataset and for the remainder the maximum read depths were $\log_2$ transformed (following the addition of 1 to all values). All RNA species, other than the miRNAs, with a maximum read depth greater than 3 were used to normalise the samples. Based upon this subset of RNAs the maximum read depth values for all of the RNAs in all of the samples were Loess normalised (*affy* package). Subsequently, miRNAs with a normalised $\log_2$ read number of 0 in all of the *Dgcr8*$^{tm1,gt1/+}$, *Dgcr8*$^{tm1,gt2/+}$, *Dgcr8*$^{gt1/tm1}$ and *Dgcr8*$^{gt2/tm1}$ cell line samples were removed from the analysis. All the normalised samples were used to construct a linear model with the *limma* Bioconductor package and subsequently *limma* was used to compare the maximum read depth for each miRNA between the *Dgcr8*$^{tm1,gt/+}$ and *Dgcr8*$^{tm1,gt/+}$ cell lines. Broadly, miRNAs with an adjusted *P*-value < 0.05 and a fold change > 2 were considered to have significantly altered expression.

2.11 Optimised transfection protocols

2.11.1 LacZ siRNA transfection

2.11.1.1 siRNAs

Stealth RNAi LacZ Reporter Control siRNA – 20μM (Invitrogen #12935-147)

Stealth RNAi Negative Control Lo GC Duplex #2 – 20μM (Invitrogen #12935-110)

2.11.1.2 Optimised RNAi protocol

Dgcr8$^{gt1/tm1}$ cells were cultured for 4 days in non-selective media. On the fourth day the cells were plated to gelatinised 6 well plates, 96 x 10^4 cells per well in 7.2 ml of non-selective media. Wells were transfected after 3 hours. 12 μl of the siRNA (20 μM) was added to 240 μl OptiMEM I (Gibco). 7.2 μl of Lipofectamine 2000 (Invitrogen) was added to further 24 μl of OptiMEM and incubated for 5 minutes at room temperature. Both solutions were mixed gently by pipetting the total volume prior to incubation. The two mixtures were combined and mixed as above. This mixture was incubated for a further 25 minutes at room temperature. The media was gently aspirated from the cells and 2.4 ml of fresh, non-selective media was added to each well. The siRNA-lipid complexes in the OptiMEM mixture were then transferred to this fresh media and the wells were mixed by pipetting the total volume very gently and by rocking the plate 20x. 5 hours later, the media was aspirated from the cells and replaced with a further 7.2 ml of non-selective media.

20 hours after the siRNA-lipid complexes were added to the cells, the cells were Xgal stained (see section 2.5.1).

2.11.1.3 LacZ siRNA transfection and slides

2.11.1.3.1 Slide preparation

Dgcr8$^{gt1/tm1}$ cells were transfected as above, but all reagents and cell numbers were scaled proportionately according to the surface area of the wells (1/12).

Slides were fixed and stained as above (see section 2.5.2.2). In addition to *Dgcr8*$^{gt1/tm1}$ cells, *Dgcr8*$^{tm1,gt1/+}$ cells prepared for Oct4 immuno-staining (see section 2.5.2) were stained alongside the transfected cells as a control. Primary antibodies were added together: LacZ (5 Prime-3 Prime, 1/100) and Oct4 (Santa Cruz – sc8628, 1/50). Secondary antibodies were also added together: Alexa Fluor 488 (Donkey anti-goat - Invitrogen) and Alexa Fluor 594 (Donkey anti-rabbit - Invitrogen). A number of control wells were included stained with either primary or secondary antibodies alone.

2.11.1.3.2 Imaging slides siRNA transfected slides

Images were taken essentially as described above (see section 2.5.2.3), however, settings were used as follows: Sequential setting 1 - Laser Line UV (405): 64%, PMT1 - ~412-489 nm, Sequential setting 2 – Laser line Visible (594): 40%, Laser line Visible (488): 100%, PMT2 - ~492-566, PMT3 - ~608-690 nm.

2.11.2 miRNA mimic transfection

2.11.2.1 miRNA mimics

miRIDIAN Negative Control #2 (Dharmacon CN-002000-01-05)

miRIDIAN mmu-miR-291-3p mimic (Dharmacon C-310470-01)

miRIDIAN mmu-miR-25 mimic (Dharmacon C-310564-01)

2.11.2.2 miRNA transfection protocol

Dgcr8$^{gt1/tm1}$ cells were transfected with miRNA mimics in a 6 well plate, according to the Optimised RNAi protocol (see section 2.11.1.2). 240 pmoles of miRNA mimic were added per well. Post-transfection media was replaced with fresh non-selective media each day. 10, 20 and 44 hours after the initiation of transfection cells were lysed for RNA. The cells were washed twice with DPBS (-$CaCl_2$ and $MgCl_2$) (Gibco) and lysed with 1ml of Trizol (see section 2.7.1). Trizol lysate was subsequently stored at -70˚C.

2.12 Optimisation of transfection conditions

The optimization experiments for the siRNA transfection of *Dgcr8*$^{gt1/tm1}$ cells were conducted in 24 well tissue culture plates and then scaled proportionately to a method suitable for 6-well transfection (See section 2.11.1.2). The details of the early pre-optimised transfection protocols are beyond the scope of this thesis, however, several methods were used to judge the efficiency of the transfection of siRNAs in these experiments. These methods are described below.

2.12.1 siGLO siRNA transfection

Briefly, *Dgcr8*$^{gt1/tm1}$ cells were transfected in suspension. Cells in a volume of 500µl of non-selective media were combined with 100µl of an OptiMEM, Lipofectamine 2000 and siGLO mix (Lamin A/C siRNA – Human, Dharmacon - D-001620-02-05). 240µl aliquots of the combined mixture were plated to individual gelatinized wells of an 8 well culture slide (Nunc). 4 hours and 40 minutes later the media was replaced with fresh non-selective media. The cells were cultured overnight, washed in 1x DPBS (-$CaCl_2$ and $MgCl_2$) (Gibco), and fixed with a few drops of 4%

paraformaldehyde solution. The cells were fixed for 20 minutes at room temperature and then the paraformaldehyde solution was removed by aspiration and replaced with DPBS (-$CaCl_2$ and $MgCl_2$) (Gibco). Slides were stored at 4˚C. Slides were subsequently washed 4x PBS and quenched with a few drops of 50 mM NH_4Cl in PBS for 10minutes. The slide wells were then washed a further 3x with PBS and finally the chambers were removed from the slide, a few drops of Vectorshield with DAPI (Vector Laboratories) were added to each slide and a cover slip was applied and fixed to the slide with nail varnish. The slides were examined with a Zeiss fluorescence microscope with Cy3 (siGLO) and DAPI filters.

2.12.2 KIF11 siRNA transfection

Dgcr8$^{gt1/tm1}$ cells in a 24 well format were transfected with a KIF11 siRNA (Silencer KIF11(Eg5) siRNA – Ambion –AM4639), a control siRNA (Negative Control #1 siRNA – Ambion – 4611G), or plated without transfection. 48 hours after transfection initiation the non-selective media in which the cells are cultured was replaced with 400 µl of Alamar Blue media. Samples were subsequently incubated in this media for 2-3hours at 37˚C, 7% CO_2). An aliquot of 100 µl of media from each well was transferred to a well of a BD Bioscience plate reader plate (BD Bioscience– 353947). The plate was plate centrifuged at 3000 rpm for 1 min at room temperature to remove bubbles from the wells. A plate reader was used to detect colorimetric changes in the media from each well (Excitation wavelength: 544nm, Emission wavelength: 590 nm, Reads: 6, Temperature: 37˚C). Transfection efficiency was judged by a ratio of the fluorescence reading of the media from a KIF11 siRNA transfected sample divided by the fluorescence reading from a control-transfected sample. The effect of transfection on cell survival was judged by dividing the fluorescence value of media from a well

of control siRNA transfected cells by the value gained from a sample from a well of non-transfected cells.

2.13 Solutions

2.13.1 General laboratory solutions

3x Sucrose/Cresol Solution

28% Sucrose

0.008% Cresol Red

1x $T_{0.1}E$

Luria-Bertani (LB) Broth

10 mg/ml Bacto-Tryptone

5 mg/ml Yeast Extract

10 mg/ml NaCl

pH 7.4

Propidium Iodide (PI) Solution

0.1% Triton X-100

50 µg/ml RNase A

20 µg/ml Propidium Iodide

10x MOPS Buffer

0.2 M MOPS

50 mM Sodium Acetate

10 mM EDTA (pH 8.0)

pH 7.0

20x Saline sodium citrate (SSC)

3 M NaCl

0.3 M Trisodium Citrate

1x Phosphate Buffered Saline (PBS)

137 mM NaCl

10 mM Phosphate Buffer

2.7 mM KCl

pH 7.4

1x Tris Borate EDTA (TBE)

89 mM Tris-borate

2 mM EDTA (pH 8)

$T_{0.1}E$

10 mM Tris-HCl (pH 8)

0.1 mM EDTA

10x NEB Buffer

670 mM Tris-HCl (pH 8.8)

166 mM $(NH_4)_2SO_4$

67mM $MgCl_2$

4% Paraformaldehyde Solution

4 % w/v Paraformaldehyde

1x PBS

pH 7.4

2.13.2 Xgal staining solutions

0.1M Phosphate Buffer

27.1 mM Monobasic Sodium Phosphate

72.9 mM Dibasic Sodium Phosphate

pH 7.3

Fix Buffer

0.1 M Phosphate Buffer (pH 7.3)

5 mM EGTA

2 mM $MgCl_2$

0.2% Gluteraldehyde

Wash Buffer

0.1 M Phosphate Buffer (pH 7.3)

2 mM MgCl2

Xgal Staining Buffer

0.1 M Phosphate Buffer (pH 7.3)

2 mM $MgCl_2$

5 mM Potassium Hexacyanoferrate(II) Trihydrate

5 mM Potassium Hexacyanoferrate(III)

1 mg/ml Xgal (Stock = 500 mg in 10 ml Dimethylformamide)

2.13.3 Western blot solutions

Protein Lysis Buffer

50 mM Tris HCl (pH 7.5)

0.5 M NaCl

1% IGEPAL CA-630

1% Sodium Deoxycholate

0.1% SDS

2 mM EDTA

COMPLETE Protease Inhibitors (Roche) (1 tablet per 10ml)

10x TBS

200 mM Tris HCl

1.37 M NaCl

pH 7.6

Western Blocking Solution

0.1% Tween20

10% w/v Powder Milk

1x TBS

Western Washing Solution

0.1% Tween20

1x TBS

2.13.4 miRNA Northern blot solutions

Denhardt's Solution (100x)

2% Ficoll 400

2% Polyvinylpyrrolidone

2% BSA

Filter with 0.2 μm syringe filter

Pre-Hybridisation/Hybridisation Solution

5x SSC

20 mM Na_2HPO_4 (pH 7.2)

7% SDS

2x Denhardt's Solution

2 mg of Sheared Salmon Sperm DNA (Sigma) preheated to 100˚C for 5 minutes

Primary Wash Solution

3x SSC

25 mM Na_2HPO_4 (pH 7.2)

5% SDS

10x Denhardt's Solution

Secondary Wash Solution

1x SSC

1% SDS

Strip Solution

0.015 M NaCl

0.1x SSC

1% SDS

2.13.5 Cell culture solutions

β- mercaptoethanol 1000x stock solution

Dilute 70 µl β-mercaptoethanol (Sigma) in 20 ml Distilled H_20 (Gibco)

Filter sterilize (0.22 µm)

L-Glutamine/Sodium Pyruvate (G/P) Solution

Mix 100 ml L-Glutamine (200 mM, 100x Gibco) with 100ml Sodium Pyruvate (100 mM, 100x Gibco)

Filter sterilize (0.22 µm)

ES Cell Culture medium

500 ml GMEM (Sigma)

50 ml Foetal Bovine Serum (FBS) (Gibco)

10 ml G/P solution

5 ml Non-Essential Amino Acids (Gibco)

0.56 ml β- mercaptoethanol solution

LIF (Quantity determined empirically by the Skarnes laboratory)

Alamar Blue Media

8.87 ml GMEM (Sigma)

1 ml FBS (Gibco)

0.2 ml G/P solution

0.1 ml Non-essential amino acids (Gibco)

11.2 µl 1000x β- mercaptoethanol solution

0.6µl LIF (Quantity determined empirically by laboratory of Dr. William Skarnes)

1.130ml Alamar Blue (Biosource)

1x Trypsin

Add 0.1 g EDTA (Sigma) to 500ml DPBS -$CaCl_2$ and $MgCl_2$ (Gibco)

Filter sterilize (0.22 µm)

Add 5 ml Chicken serum (Gibco)

Add 10 ml 2.5% Trypsin (Gibco)

Aliquot and store at -20˚C

(2x Trypsin, as above, but double volume of Trypsin added)

0.1% Gelatin

Add 25 ml 2% Bovine gelatin solution to 500 ml DPBS -$CaCl_2$ and $MgCl_2$ (Gibco)