CHAPTER 2

2 MATERIALS AND METHODS

2.1 Cell culture

2.1.1 Embryonic (ESC) and Trophoblast (TSC) stem cell lines

JM8 *wild-type* line is derived from B57BL/6N blastocyst (Pettitt, Liang et al. 2009).

AB2.2 *wild-type* line is derived from 129S6 blastocyst (McMahon and Bradley 1990).

JM8-R26-Cas9 line was a gift from Dr. Kosuke Yusa's lab. It was generated by integration of an *EF1α-Cas9-IRES-NeoR* cassette into the Rosa26 locus of *wild-type* JM8 (Tzelepis, Koike-Yusa et al. 2016). The *Cas9* gene in this construct is humanised and codon-optimised with a Nuclear Localisation Sequence (NLS) tag at the C-terminus.

All subsequent knockout lines generated in the present work were derived from either JM8-R26-Cas9 or AB2.2.

The Trophoblast Stem Cell (TSC) F4 *wild-type* line was a gift from Dr. Janet Rossant's lab and was derived from ICRxICR blastocyst.

2.1.2 Cell culture conditions

ESC and TSC lines were maintained at 5% CO_2 and 37°C.

In standard conditions, ESCs were cultured in M15Lif medium in gelatinised plates. For chimera assay injections, cells were maintained in M15 medium, on a layer of irradiated feeder fibroblasts (SNL76/7) for at least three passages (Ramírez-Solis, Davis et al. 1993). Medium was changed daily.

Where indicated, ESCs were converted from standard conditions to different culture systems, by maintenance in 2iLif or EPSCM media for at least three passages.

For maintenance of TSCs, cells were cultured in TSC media, on a layer of irradiated mouse embryonic fibroblasts prepared from E14.5 embryos (MEFs) (Tanaka, Kunath et al. 1998). When specified, TSCs were converted to TX medium for at least three passages, also on irradiated MEFs (Kubaczka, Senner et al. 2014). Medium was changed every two days.

2.1.2.1 Media composition

The different media formulations used throughout this work were as follows:

1. M15 medium: DMEM (Lonza) supplemented with 15% fetal bovine serum (Gibco), 100 μM β-mercaptoethanol, 1x non-essential amino acids and 2 mM L-glutamine.
2. M15Lif medium: M15 medium supplemented with 100 U/mL recombinant human leukemia inhibition factor (LIF) (Millipore).
3. 2iLif medium: 1:1 mix Neurobasal medium and DMEM/F12, supplemented with 1x N2, 1x B27 (all from Gibco), 100 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL LIF, 1 μM of the MEK inhibitor PD0325901 (Stem Cell Institute) and the 3 μM of the GSK3β inhibitor CHIR99021 (Stem Cell Institute).
4. TSC medium: RPMI-1640 (Gibco), 20% fetal bovine serum (Gibco), 1x Penincillin-Streptomycin-Glutamine, 1 mM sodium pyruvate, 100 μM β-mercaptoethanol supplemented with 25 ng/mL Fgf4 (Biolegend) and 1 μg/mL Heparin (Sigma).
5. TSC terminal differentiation medium: TSC medium without Fgf4 and Heparin.
6. TX medium: DMEM/F12 supplemented with 1x N2 (Gibco) as source for insulin, holo-transferrin and sodium selenite, 1x Penincillin-Streptomycin-Glutamine, 64 μg/mL vitamin C (Sigma), 25 ng/mL Fgf4 (Biolegend), 1 μg/mL Heparin (Sigma) and 2 ng/mL human recombinant TGF-β1 (Preprotech).

2.1.2.2 Antibiotic selection

Antibiotics used for selection were:

1. Puromycin (1.5 - 3 μg/mL), for selection of Puro expression. Concentrations were adjusted depending on the batch of puromycin in use.

2. G418 (Geneticin) (500 μg/mL), for selection of Neo expression. This high concentration was used to select for high expression of Cas9-IRES-Neo.
3. Blasticidin S (10 μg/mL), for selection of Bsd expression.

2.1.3 ESC and TSC passaging and cryopreservation

Cells were passaged when about 80% confluent, by washing with PBS followed by treatment with trypsin for 5 minutes (TSCs) or 15 minutes (ESCs), at 37°C. Trypsin was inactivated with serum-containing media. ESCs were routinely passaged 1:4 every 2 - 3 days, whereas TSCs were passaged 1:20 every 3 – 4 days.

For cryopreservation, upon harvesting, cells were resuspended in 500 μL freezing media (90% fetal bovine serum and 10% DMSO) and placed at -80°C overnight, before transfer to liquid nitrogen or -150°C for long term storage.

2.1.4 *In vitro* differentiation towards trophoblast lineage

Upon optimisation of differentiation conditions (discussed on chapter 3), the protocol adopted for differentiation of ESCs to TSC lineage consisted on plating 200,000 ESCs onto gelatinized 12-well plates with a layer of irradiated MEFs. Cells were cultured in either TSC medium (default) or TX medium, when specified. Medium was changed every other day and differentiation carried out for 14 – 18 days. For plating in different formats, cell density and media volume were scaled proportionally to culture area.

For terminal differentiation, cells were cultured in gelatinised plates without irradiated MEFs and maintained with TSC terminal differentiation medium (withdrawal of Fgf4 and Heparin).

2.1.5 ESC transfection

For all transfections, ESCs were fed with fresh media 2 - 3h in advance. Media was changed the next morning and appropriate antibiotic selection (see section 2.1.2.2) started 48h post-transfection.

2.1.5.1 Lipofection

Transfection of ESCs was carried out using Lipofectamine LTX Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, considering the reaction for 6-well plates, a total of 1.5 µg of DNA was mixed with 4 µL of Plus reagent and 500 µL Opti-MEM (Gibco) and incubated 5 minutes at room temperature. 12 µL of Lipofectamine LTX were then mixed with the DNA solution and incubated at room temperature for at least 5 minutes. Next, 1 to 1.5 million cells resuspended in 500 µL of M15Lif were plated in a gelatinised well, immediately followed by drop-wise addition of the lipofectamine:DNA complexes and topped up with 1 mL M15Lif.

2.1.5.2 Nucleofection

For nucleofection, 1 to 1.5 million ESCs were resuspended in 100 µL of Opti-MEM and mixed with 1.5 µg of DNA. Cells were then electroporated using Amaxa Nucleofector (Lonza), with programme A-023, followed by addition of 1 mL of M15Lif media to the cuvette and transfer to gelatinised 6-well. Wells were topped up with an extra 1 mL of media.

2.2 Molecular Biology

2.2.1 Molecular cloning

2.2.1.1 Gibson assembly of *Elf5* targeting construct

The *Elf5* targeting construct (pl253-Elf5-T2A-H2B-Venus-LoxP-PGK-Bsd) was generated using pl253 backbone plasmid (Liu, Jenkins et al. 2003) digested with *NotI* and *BamHI*. The left homology arm was ordered as a gene block that adds a *T2A* sequence at the end of *Elf5* CDS, and the remaining fragments were PCR amplified as described in Methods Table 2.1. pl253-Oct4-2Avenus-2A-BSD template was a kind gift from Dr. Xuefei Gao and pl514 from Dr. Pentao Liu (unpublished). All PCR products were purified using Agencourt AMPure XP beads prior to Gibson assembly following the manufacturer's instructions (NEB). Briefly, 50 ng of digested plasmid were mixed with 30 ng of each fragment and Gibson Master mix and incubated for 1 hour at 50°C.

NEB10β cells were used to transform the product. Cloning was confirmed by restriction analysis and Sanger sequencing.

2.2.1.2 Gibson assembly of *Rnf2-TY1* overexpression construct

pKLV-flipedU6gRNA_PB_BbsI_PGKpuro2ABFP (Metzakopian, Strong et al. 2017) was used as backbone plasmid, upon double digestion with *BbsI* and *KpnI* restriction enzymes. Gibson cloning was then performed as described in the previous section, using fragments generated according to Methods Table 2.2.

2.2.1.3 gRNA design

gRNA sequences were designed using online tools developed by the Zhang lab (MIT) (http://crispr.mit.edu; http://www.genome-engineering.org) or the WTSI Genome Editing database (https://www.sanger.ac.uk/htgt/wge/) (Hodgkins, Farne et al. 2015). These tools allow the selection of genomic target sites followed by a 5'-NGG or 5'-NAG PAM sequence with minimal likelihood of off-target genome modifications.

2.2.1.4 gRNA cloning

gRNA sequences used in this work can be found on Methods Table 2.4.

For *Elf5*, *Oct4* and *Dnmt1* targeting gRNAs, the backbone plasmid used was pKLV-flipedU6gRNA_PB_BbsI_PGKpuro2ABFP (Metzakopian, Strong et al. 2017).

All remaining gRNAs were cloned in pLVPB-U6-sgRNAv2fl-shortccdB-PGK-Puro-BFP backbone plasmid, a gift from Dr. Mathias Friedrich. This plasmid has an improved gRNA scaffold, demonstrated to result in higher knockout efficiency compared with the same gRNA expressed in conventional scaffold (Chen, Gilbert et al. 2013, Dang, Jia et al. 2015, Tzelepis, Koike-Yusa et al. 2016). This is also equivalent to the gRNA library version 2 used in this work (Tzelepis, Koike-Yusa et al. 2016).

For each gRNA, oligos were ordered from IDT, containing the 20nt gRNA sequence and overhangs compatible with *BbsI* digestion of each backbone plasmid. Sense and antisense oligos (10 μM each) were mixed with T4 ligase buffer in 100 μL reaction. The mixture was incubated at 98 °C for 5 min followed by ramp down to 25°C at 5 °C min−1. Annealed products were diluted 1:100 and used for ligation to *BbsI*-digested backbone plasmid, using T4 DNA ligase (NEB). All cloned gRNAs were sequence verified by Sanger sequencing.

2.2.2 Nucleic Acid Extraction

2.2.2.1 Plasmid DNA preps

In default conditions, DNA plasmids were propagated using NEB10β chemically competent cells (NEB) grown in LB medium with appropriate antibiotic selection.

Minipreps and midipreps were obtained using NucleoSpin Plasmid kit and NucleoBond Xtra Midi EF kit, respectively (Macherey-Nagel), as per manufacturer's instructions.

2.2.2.2 Genomic DNA extraction

To genotype clones picked from ESC targeting experiments, media was aspirated from confluent 96-well plates and cells were frozen at -20°C. Upon thawing, 50 μL of lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% Sarcosyl and 1 mg/mL proteinase K) were added to each well followed by 3-hour incubation at 65°C. 150 μL of precipitation buffer (absolute ethanol with 150 mM NaCl) were added per well and the plate was centrifuged for 20 minutes at full speed in an Eppendorf 5804 R centrifuge. Supernatant was decanted and genomic DNA washed twice with 70% Ethanol. Pellets were allowed to dry and resuspended in 50 μL of water. Typically, 1 μL of extracted gDNA was used for genotyping PCR.

For larger cell numbers, genomic DNA was extracted with QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions.

2.2.2.3 RNA extraction

In standard conditions, RNA was isolated using the RNAeasy Mini Kit (Qiagen), according to the manufacturer's instructions.

For samples from low cell number (less than 0.25 million cells), RNA was extracted using the Arctarus PicoPure RNA Isolation kit (Applied Biosystems), as per the manual guidelines.

2.2.3 Genotyping

Primers used for the different genotyping reactions can be found on Methods Table 2.3.

2.2.3.1 Standard PCR genotyping

To genotype *Elf5* targeting clones, PCR was performed using ThermoScientific Extensor Long PCR Reddy Mix according to manufacturer's instructions.

2.2.3.2 Indel detection

Surveyor Assay

The region surrounding gRNA cutting site was amplified by PCR using KAPA-HiFi Hot Start (usually around 1kb long, with the cutting site in the middle). PCR products were purified with Agencourt AMPure XP beads in a PCR-product:bead ratio of 1:0.7 and normalised to 20 ng/μL. Surveyor Assay was performed as described in (Ran, Hsu et al. 2013). Purified PCR products were mixed with LongAmp Taq 1x buffer in a total volume of 20 μL for DNA heteroduplex formation PCR. The hybridized product was then incubated with Surveyor Nuclease (IDT), according to manufacturer's instructions and the reaction was incubated for 1 hour at 42°C. The product was analysed on a 2% agarose gel.

Tracking of Indels by DEcomposition (TIDE)

Similarly to Surveyor Assay, the region surrounding gRNA cutting site was amplified by PCR using KAPA-HiFi Hot Start and purified with Agencourt AMPure XP beads in a PCR-product:bead ratio of 1:0.7. A wild-type sample for the same region was included as a control. Products were Sanger sequenced and results were analysed using the online TIDE resource (https://tide.deskgen.com/)(Brinkman, Chen et al. 2014).

2.2.4 cDNA preparation

2.2.4.1 “Standard” Reverse Transcription

First strand cDNA was generated using the QuantiTect Reverse Transcription Kit (Qiagen), according to manufacturer’s instructions. Briefly, 500 – 1000 ng of total RNA were used for elimination of genomic DNA followed by reverse transcription for 1h at 42°C. The reaction was inactivated for 3 min at 95°C. For RT-qPCR analysis, cDNA was diluted 1:100 and 3 μL were used per reaction.

2.2.4.2 Low input samples – adaptation Smartseq2 protocol

For samples with low cell number (less than 0.25 million cells), cDNA preparation was a limiting factor when using standard reverse transcription protocols. To work with these, an adaptation of single-cell Smart-seq 2 method (Picelli, Faridani et al. 2014) was performed. After RNA extraction with the PicoPure kit, 50 ng of total RNA (in a total volume of 3.5 μL) were used for oligodT annealing with 1 μL of dNTP mix and 1 μL of oligodT primer (100 μM stock), incubated for 3 minutes at 72°C and immediately placed on ice.

The first strand reaction was performed as described in (Picelli, Faridani et al. 2014), using Superscript II enzyme (Invitrogen) and the same PCR parameters. The product from this reaction was diluted 1:3 and 20 μL were used for preamplification with KapaHiFi HotStart Ready Mix (Kapa Biosystems), which was then carried on as described in the original paper, with 15 cycles. The number of cycles for preamplification was previously optimised by analysis of PCR product on agarose gel.

cDNA was purified using Agencourt AMPure XP beads in a PCR-product:bead ratio of 1:0.7 and analysed on a Agilent High Sensitivity DNA Bioanalyzer Chip (Agilent).

For all RT-qPCR reactions using cDNA prepared with this method, appropriate controls were made with similar cell numbers to limiting samples.

2.2.5 RT-qPCR

For real-time PCR, TaqMan Gene Expression Assays (Life Technologies) (Methods Table 2.5) and ABsolute Blue qPCR ROX Mix (ABgene) were used. Quantitative PCR

was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems). Gene expression was determined relative to GADPH gene using the ΔCt method. Data are generally shown as the mean and standard deviation (s.d.) of three technical replicates.

2.2.6 *Elf5* promoter DMR analysis

DMR analysis was performed in collaboration with Dr. Sandeep Rajan.

Genomic DNA for bisulfite conversion was extracted using QIAamp Mini DNA Kit (Qiagen). 500 ng of gDNA were used per bisulfite treatment with EZ DNA Methylation StartUP Kit (Zymo), according to the manufacturer's instructions. The *Elf5* promoter region was then PCR amplified using Zymotac and primers described on Methods Table 2.6 (PCR parameters: annealing 52°C for 35 seconds, extension 72°C for 35 seconds, 40 cycles). PCR products were cloned into pGEM-T Easy Vector (Promega) and transformed in NEB10β cells. Cells were plated on LB-ampicillin plates with X-Gal for blue/white selection. For each sample, ten white colonies were sequenced.

2.3 Flow cytometry and fluorescent-activated cell sorting (FACS)

For flow cytometry, cells were harvested by trypsinization and analyzed accordingly to the fluorescent reporter combinations of interest using either the LSRFortessa instrument or Cytoflex (both BD Biosciences). For the LSRFortessa instrument, the different fluorescent reporters were analyzed with the following parameters: BFP signal obtained using the 405nm laser, and 450/50 filter; Venus / AF488 signal obtained using the 488nm laser, and 530/30 filter; mCherry / AF594 signal obtained using the 591nm laser, and 610/20 filter. For the Cytoflex, the following parameters were used: BFP detected through the 405nm laser, and 450/45 BP filter; Venus / AF488 detected through the 488nm laser, and 525/40 BP filter; mCh / AF594 detected through the 488nm laser, and 610/20 BP filter; PE detected through 488 nm laser and 585/42 BP.

For FACS, cells were harvested by trypsinization and analyzed using either the BD Influx Cell Sorter (BD Biosciences) or the MoFlo (Beckman Coulter). The parameters in both these instruments were equivalent to the LSRFortessa.

2.4 Lentivirus methods and genome-wide CRISPR/Cas9 screening

2.4.1 Lentivirus Production

Lentivirus was produced by transfection of HEK-293FT cells (a gift from Dr. Kosuke Yusa's lab) using Addgene packaging plasmids psPAX2 and pMD2.G and lipofectamine LTX. HEK-293FT cells were cultured in D10 medium (DMEM supplemented with 10% FBS and 1x glutaMAX (Gibco)).

To produce concentrated virus, a 15-cm plate format was generally used. Cells were grown on gelatinised 15-cm plates and split 1:3 one day ahead of transfection to achieve about 70-80% confluency on the day. For transfection, 7.5 µg of lentiviral transfer vector (containing cloned gRNAs as described in section 2.2.1.4) were mixed with 18.5 µg of psPAX2 and 4 µg of pMD2.G in 7.5 mL of optiMEM media followed by the addition of 30 µL of PLUS reagent and incubation for 5 minutes at room temperature. 90 µL of Lipofectamine LTX reagent were then added to the mix and incubated for 30 minutes. Medium was aspirated from the plates and the Lipofectamine:DNA complexes were added dropwise and topped-up with 12.5 mL of D10 medium. Medium was changed the following morning to fresh D10 (45 mL) and the supernatant was harvested 48 hours later. Supernatant was filtered through 45 µm surfactant-free cellulose acetate filters and lentivirus were concentrated by centrifugation at 6.000 g, overnight at 4°C. Each pellet obtained from a 15-cm plate was resuspended in 400 µL of PBS, aliquoted and stored at -80°C.

Each batch of concentrated lentivirus produced was tested by titration in order to determine the amount needed to achieve MOI 0.3.

2.4.2 ESC transduction and Lentivirus titration

The method described considers a 24-well plate format and was generally used for lentivirus titration and screening validation experiments. All other scale-up reactions were performed directly proportional to culture area.

For lentivirus titration, a serial dilution was prepared ranging from 1:10 to 1:10.000 in a total volume of 100 µL M15Lif supplemented with 8 µg/mL of polybrene (Sigma). ESCs

were harvested and resuspended at a density of 300.000 cells per 400 μL of M15Lif supplemented with 8 μg/mL of polybrene (Sigma). Per transduction, 400 μL of media-containing cells was incubated with the appropriate lentivirus dilution and incubated 30 minutes at 37°C before plating onto gelatinised 24-well plates. Media was changed the next morning and lentivirus infection efficiency was determined 48 hours post-transduction using flow cytometry.

For ESC transduction the protocol was as above, using the virus dilution determined for 20-30% infection rate. Typical virus dilution for concentrated virus was 1:500 – 1:1.000.

In the screening validation experiments, lentiviruses were produced in 6-well plate format and the supernatant (2 mL per well) was directly used to infect ESCs (either fresh or thawed from -80°C). Typically using 1:1 mix of supernatant and M15Lif medium-containing ESCs yielded 15 - 30% transduction efficiency.

2.4.3 Genome-wide mouse gRNA lentiviral library

The genome-wide mouse gRNA lentiviral library (version 2) used in this work was a gift from the Yusa Lab at the WTSI (Tzelepis, Koike-Yusa et al. 2016).

2.4.4 Genome-wide ESC mutant libraries and screening

90 million ESCs (JM8-R26-Cas9-Elf5-T2A-H2BVenus, from now on referred to as *Elf5::Venus* ESCs for simplification) were transduced with the gRNA lentiviral library at an MOI of 0.3. Two independent transductions were performed, generating two independent mutant ESC libraries. Two days post-transduction, puromycin was added to the cultures for selection of gRNA-carrying cells. For each independent transduction, two differentiation screens were conducted as biological replicates. Given that the phenotype studied is tightly connected with ESC self-renewal, we tried to maximize the diversity of ESC mutant libraries at the start of differentiation by setting up differentiations at different timepoints post-transduction. Screening 1.1 started 7 days post-transduction, and its biological replicate 1.2 started 9 days post-transduction (one extra passage of the ESC mutant library). Screening 2.1 started 5 days post-transduction and 2.2, 7 days. For each screen, at day zero of differentiation, 48 million mutant ESCs were seeded into a total of twenty 12-well plates with MEFs (200.000 ESC cells per well) on TSC medium.

Media was changed every two days. At day 14 of differentiation, cells were harvested by trypsinization and Venus positive cells were sorted. To verify mutant ESC library quality, a sample of each replicate was collected at day zero for differentiation. For posterior gRNA enrichment analysis, at day 14 we collected three populations: unsorted, Venus negative and Venus positive. Genomic DNA was extracted from all these samples and used for PCR templates.

2.4.5 Illumina sequencing of gRNAs

For sequencing of the gRNAs in the different screening populations, we proceeded as described in (Koike-Yusa, Li et al. 2014). The region containing the gRNA was amplified using 1 µg of genomic DNA and primers (gLibrary-HiSeq_50bp-SE U1 (ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGTGGAAAGGACGAAACA) and –L1 (TCGGCATTCCTGCTGAACCGCTCTTCCGATCTCTAAAGCGCATGCTCCAGAC) (Koike-Yusa, Li et al. 2014)) with Q5 Hot Start High-Fidelity 2x Master Mix and the following conditions: 98°C for 30 s; 27 cycles of 98°C for 10 s, 60°C for 30 s and 72°C for 15 s; and the final extension 72°C for 2 min. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen). Two hundred picograms of the purified products were used for PCR enrichment with KAPA HiFi HotStart ReadyMix using primers iPCRtagT1 to iPCRtagT16 and PE 1.0 (Illumina) and conditions as stated in (Koike-Yusa, Li et al. 2014). Lastly, the PCR products were purified with Agencourt AMPure XP beads in a PCR-product:bead ratio of 1:0.7. Purified libraries were quantified and sequenced on 2 lanes of HiSeq2500 by 75-bp paired-end sequencing.

2.5 Immunostaining

Methods Table 2.7 describes the list of antibodies used in this work.
All immunostainings were carried out at room temperature unless stated otherwise.

2.5.1 Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes and subsequently blocked and permeabilised by 20 minutes incubation in blocking buffer (3% serum* in PBS with 0.1% Triton X100 and 1% BSA). Cells were incubated with primary antibody

overnight at 4°C. After washing with PBS containing 0.1% Triton X100, cells were incubated with the appropriate secondary antibody (diluted 1:1000 in blocking buffer) for 1 hour in the dark. Cells were washed and counter stained with DAPI for 5 minutes.

Note: * Serum from the species where the secondary antibody was raised in.

2.5.2 Embryo Immunostaining

Embryo staining protocol was adapted from (Nichols, Silva et al. 2009). Embryos were fixed in 2.5% PFA in PBS for 15 minutes (blastocyst stage) or 30 minutes (E6.5 embryos) and washed in PBS/PVP solution (PBS containing 3 mg/mL polyvinylpyrolidone). Permeabilisation was performed by 2 hours incubation with 0.25% Triton X in PBS/PVP buffer, followed by blocking for 1h in blocking solution (PBS/PVP containing 0.1% BSA, 0.01% Tween20 and 2% donkey serum). They were then incubated with appropriate primary antibody dilutions (in blocking solution) overnight at 4°C. Embryos were rinsed three times in blocking solution for about 15 minutes each, followed by incubation with secondary antibodies (diluted 1:500 in blocking solution) for 1 hour in the dark. Embryos were again washed three times and moved through a series of 50%, 75% and 100% ProLong Gold Antifade Mountant with Dapi (Life Technologies) before mounting in a small drop of mounting media, covered with a coverslip and sealed with nail varnish.

2.5.3 Image acquisition

For embryo imaging, stained samples were analysed with a Leica DM5000B microscope equipped with narrow band-pass filters for Dapi, Cy5.5, Cy3.5 and FITC fluorescence. Images were acquired with a monochrome digital camera (ORCA-03G, Hamamatsu). Immunocytochemistry and live cell imaging were acquired with either Olympus IX81 or Zeiss Vert A1 microscopes. All images were processed with ImageJ (Schneider, Rasband et al. 2012) and Adobe Photoshop.

2.5.4 Cell surface Immunostaining for flow cytometry analysis

Cells were harvested and washed once with staining buffer (PBS, 0.5% BSA). Approximately 1 to 2 million live cells were used per stain. Cells were blocked with 50

μL of Mouse BD Fc Block (BD Biosciences), diluted 1:200 in staining buffer, for 15 minutes. Primary antibody was directly added to each sample (1:10 dilution) and incubated for 1 hour in the dark. Cells were washed twice with staining buffer and analysed by flow cytometry.

2.5.5 Intracellular Immunostaining for flow cytometry analysis

The protocol was carried out at room temperature unless stated otherwise.

Upon harvesting, cells were fixed in IC Fixation Buffer (Invitrogen) for 10 minutes at 4°C. Cells were washed twice with PBS and permeabilised with permeabilisation buffer (PBS, 0.1% Tween) for 20 minutes. Blocking was performed with PBS, 0.3M glycine and 10% goat serum, for 30 minutes, followed by incubation with primary antibody diluted in permeabilisation buffer, 1 hour in the dark. Cells were washed twice and incubated with secondary antibody (1:2000 dilution in permeabilisation buffer) for 1 hour in the dark followed by two washes in FACS buffer (PBS, 2%FBS) and analysed by flow cytometry.

For Cdx2 staining better profiles were observed using the FoxP3 buffer system (eBioscience), following the same protocol as above, but using FoxP3 Fixation/Permeabilisation buffer for fixation and FoxP3 permeabilisation buffer in the remaining steps.

2.5.6 Western Blot

2.5.6.1 Nuclear Extracts

Nuclear extracts (NE) were prepared from ESCs grown in 10-cm plates. Cells were washed twice with cold PBS and scraped from the plate followed by centrifugation for 4 minutes at 300 g. Cell pellets were resuspended in 1 mL of nuclear extract buffer A (10 mM KCl, 20 mM HEPES pH7.9, 1 mM EDTA, 1 mM Dithiothreitol and 1x Protease inhibitor cocktail) and incubated on ice for 2 hours. NP-40 was added at 1% final concentration and tubes were vortexed for 30 seconds before pelleting nuclei by centrifugation at 2000 g for 5 minutes. Supernatants were aspirated and cell nuclei resuspended in 200 μL of Nuclear extract buffer B (400 mM NaCl, 10 mM EDTA, 25% glycerol, 1 mM Dithiothreitol and 1x Protease inhibitor cocktail) and incubated on ice for 1 hour. Nuclear debris were

pelleted by centrifugation for 10 minutes at 12000 g and supernatant containing NE was collected in a new tube and stored at -20°C.

2.5.6.2 Western Blot

Protein was quantified using BCA assay (Thermo Scientific). 40 µg total protein were resolved by SDS-PAGE in a 4 - 15% gradient Mini Protean TGX gel (BioRad), transferred onto PVDF membranes and probed with antibodies described in Methods Table 2.7 | List of antibodies.

2.6 RNAseq library preparation and NGS sequencing

RNAseq library preparation was performed by the Sample Management pipeline.

RNA samples were normalised to 100 ng / 50 µL. Library preparation was performed using NEB Ultra II RNA custom kit, on an Agilent Bravo WS automation system. cDNA libraries were indexed using KapaHiFi Hot start mix and IDT dual indexed tag barcodes on Agilent Bravo WS automation system, in a PCR reaction with 14 cycles. Libraries were then purified using Agencourt AMPure XP SPRI beads on Caliper Zephyr liquid handling platform. They were quantified with Biotium Accuclear Ultra high sensitivity dsDNA Quantitative kit and pooled in equimolar amounts on a Beckman BioMek NX-8 liquid handling platform. Pooled libraries were quantified on an Agilent Bioanalyzer and normalised to 2.8 nM for sequencing. Libraries were then loaded on six lanes of an Illumina HiSeq4000.

2.7 Embryo work

All animal experiments were performed in accordance with the UK's 1986 Animals and Scientific Procedures Act and local institute ethics committee regulations.

Preparation of embryos and foster recipients as well as ESC injections were carried out in collaboration with Dr. Guocheng Lan and Dr. Xiangang Zou at Cancer Research UK Cambridge Institute.

2.7.1 Preparing embryos and foster recipients

To generate 8-cell stage embryos, CD1 females were super-ovulated by intraperitoneal injection of Pregnant Mare Serum Gonadotropin (7.5 IU each) followed by injection of human Chorionic Gonadotropin (7.5 IU each) 48 hours later. Vaginal plugs were checked the next morning (0.5 days post coitum, dpc), and 8-cell embryos were collected at 2.5 dpc and cultured in KSOM medium with 3 mg/mL BSA in an incubator with 5% CO_2 at 37°C.

For foster recipient preparation, CD1 females were mated with vasectomized C57BL6/JCBA F1 males two days before the planned injection date. At the following day, females were checked for copulation plugs. Plugged females were used as foster recipients for embryo transfer.

2.7.2 ESC microinjection into 8-cell embryos for chimera assays

To follow ESC contribution in chimera assays the injected cells were stably transfected with a PB-EF1α-H2B-mCherry-PGK-Puro construct, using Lipofection.

For chimera generation, 6-8 cells were microinjected into 8-cell embryos at room temperature, in M2 medium (Sigma). The injected embryos were then cultured *in vitro* in a mix 1:1 of KSOM and ESC culture media and allowed to develop to the blastocyst stage.

For *in vitro* chimera assays, cultured embryos were allowed to progress to the hatched blastocyst stage. Contribution was then assessed by live imaging, followed by embryo fixation for immunostainings.

For *in vivo* chimera assay, a maximum of 20 injected blastocysts were transferred into both uterine horns of a foster recipient. Embryos were then collected at E6.5 for analysis of chimera contribution.

2.8 Computational methods

2.8.1 CRISPR/Cas9 screening analysis

Screening analysis was performed in collaboration with Dr. Hannes Ponstingl and Nikolaos Patikas. gRNA counts were obtained with an *in-house* written software that

counts based on exact match to the reference gRNA library (Tzelepis, Koike-Yusa et al. 2016). Enrichment analysis was performed with MAGeck software version 0.5.4 (Li, Xu et al. 2014). Pathway analysis was obtained using GSEA online software and Molecular Signatures Database (MSigDB) v6.2 (Subramanian, Tamayo et al. 2005). Protein interaction analysis was done with STRING database (Szklarczyk, Morris et al. 2017).

2.8.2 RNAseq analysis

RNAseq analysis was performed in collaboration with Nikolaos Patikas.

Reads were mapped using STAR [] v2.5.3 and the reference genome GRCm38.p5 from ENSEMBL. Gene vectors were generated during mapping using the "--quantMode GeneCounts" option and the *Mus Musculus* GRCm38 version 91 gene annotations. QC was carried out by inspecting the uniquely mapped reads to the reference genome and by performing Principal Component Analysis on the log-normalized count vectors. Differential expression analysis was carried out using the DESeq2 (Love, Huber et al. 2014) with lfcShrink option. Genes were considered upregulated if displaying FDR < 0.1 and $\log_2$(Fold-change) > 1. Conversely, downregulated genes were defined as those showing FDR < 0.1 and $\log_2$(Fold-change) < -1. Downstream gene set enrichment analysis was performed using GSEA online software and Molecular Signatures Database (MSigDB) v6.2 (Subramanian, Tamayo et al. 2005) with Hallmark Collection (Liberzon, Birger et al. 2015) and Biological Processes GO-term analysis.

2.8.3 CHIPseq data analysis (Published dataset)

CHIPseq analysis was performed in collaboration with Dr. Vijaya Baskar.

The ChIPseq data generated by Wang *et al* (Wang, Gearhart et al. 2018) for Pcgf1, Bcor, Rnf2 and Input DNA were downloaded from Gene Expression Omnibus (GSE104690). The reads were aligned to the human genome (hg38) using Burrows-Wheeler Aligner (Li and Durbin 2009). The peaks for PCGF1, BCOR and RNF2 were the regions where the aligned reads were significantly ($p<0.05$) enriched compared to the Input DNA, according to MACS2 (Zhang, Liu et al. 2008). These peaks were merged to form a master set of 38978 binding regions using BedTools (Quinlan and Hall 2010) and mapped to the nearest genes using ChIPseeker (Yu, Wang et al. 2015).

2.9 Methods Tables

All primer sequences are 5’ to 3’.

Methods Table 2.1 | Primers for Gibson assembly of *Elf5* targeting vector. Highlighted in bold are the overlapping regions for the different fragments.

PCR fragment	Primer Name	Primer Sequence	Template
H2B-Venus	Elf5_F2_FOR	GACCGGGGGGACCAG	pl253-Oct4-2Avenus-2A-BSD
	Elf5_F2_REV	**TGTGATATGGCTGATTATGATCATTACTTA**CTTGTACAGCTCGTCCATGC	
bovine PA	Elf5_F3_FOR	TAAGTAATGATCATAATCAGCCATATC	pl253-Oct4-2Avenus-2A-BSD
	Elf5_F3_REV	**AATTGGGCTGCAGG**AGATCCAGACATGATAAGATACATTG	
LoxP-PGK-Bsd	Elf5_F4 _FOR	**TATCATGTCTGGATC**TCCTGCAGCCCAATTC	PL514
	Elf5_F4_ REV	**CAACAACAGAAATCC**GATCCCCTCGAGGGAC	
Elf5-RH Arm	Elf5_F5_FOR	**TCCCTCGAGGGGATC**GGATTTCTGTTGTTGGAAACAATCAGATC	genomic DNA (JM8 ESC)
	Elf5_F5_REV	**TCTTGAAAACCACACTGCTCGACTCTAGA**GATAGCCACCAGTGAGGTGAAG	
Elf5-LH Arm	Gene Block (Adds T2A sequence, underlined, at the end of Elf5 CDS)	**TAGGGCGAATTGGAGCTCCACCGCGGTGGC**GGCCGCCCCCTGAAGAGAACTGTGGCATCCTGGAATGGGAAGACAGGGAGCAGGGCATTTTCCGAGTGGTTAAGTCAGAAGCCCTGGCAAAGATGTGGGGACAAAGGAAGAAGAATGACAGGATGACGTACGAGAAGCTGAGCCGAGCCCTGAGGTAAGTGGGCAGCAACGGACGCAGTGAACCTGCAAAGACAAGTTATATAAAATAGCGGGGCCGGGGAAACCCACACTCTGTTCAAAGCTTCCCCTCCCCCGCTTCATATGAAAGGGTCACGAGCTAAACCTCTTCTTAGTTTCAGATGGGATTATGGGTGGTAAAGTGACCCACCATCCTTTTTTGGCCTTGGCAGCATTCCCTGGGGGGGATCACCCTAAACCCAAGCTGTGGTGCACACCAGCACACAAACGCACGCTCAACTGACTGTGGAGAGAGAGGTTCAGCCTGTGAAGTGTCTGGGAAGGAAGGGTTTAAGTGTAAGCTGATAACGGGTGTGAAAATACCAGGGTAAGTAGAGCCCGGGCTATCACCTGAGAGAGCCAGGAGTGTTTATATATGCTGTTGTTTTTCTTGTGGTGCAAACCTTCTCTGGATTTCTCTCCTTTGCTTGCTTTCACAGATACTACTATAAAACGGGAATTCTGGAGCGGGTTGACCGGAGGTTAGTGTACAAATTTGGAAAGAACGCGCACGGGTGGCAGGAAGAGAAACTCGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCCGGACCGGGGGGGAC**CAGAGCCAGCGAAGTCTG**

Methods Table 2.2 | Primers for Gibson assembly of *Rnf2-TY1* overexpression construct. Highlighted in bold are the overlapping regions for the different fragments. Cloning carried out by Dr. Sandeep Rajan.

PCR fragment	Primer Name	Primer Sequence	Template
EF1α promoter	Fragment1_F	**GGACTAGCCTTATTTTAACTTGCTATTTCTAGCTC**GCTCCGGTGCCCGTCAGTG	EF1α_Lig4_expressior vector (designed *house*)
	Fragment1_R	**TTGAGTTCCATTTGTCTGCACAGCCTGAGACAT**GTCGACGGTGGCGGCACC	
Rnf2 CDS 1	Fragment2_Nested_F	ATGTCTCAGGCTGTGCAGACAAAT	cDNA JM8 ESC
	Fragment2_Nested_R	TTTGTGCTCCTTGGTGGGTGCAT	
Rnf2 CDS (primer adds 2xTY1 tag, underlined)	Fragment2_F	**GATCTATTTCCGGTGCCGCCACCGTCGAC**ATGTCTCAGGCTGTGCAGACAAAT	Rnf2 CDS 1 fragment
	Fragment2_R	**GGACCCGCCGCC<u>AGCATCCAGAGGATCCTGATTTGTATGGACTTCGGCGTCCAGGGGGTCCTGGTTGGTGTGCACCTC</u>TCCTCCGGACCCGCCGCC**TTTGTGCTCCTTGGTGGGTGCATA	
T2A-Puro^{R} (primer adds T2A sequence, underlined)	Fragment3_F	**CATACAAATCAGGATCCTCTGGATGCTGGCGGCGGGTCCGGAGGA<u>GAGGGCAGAGGAAGTCTCCTAACATGCGGTGACGTGGAGGAGAATCCTGGCCCA</u>**ATGACCGAGTACAAGCCCACG	pKLV-flipedU6gRNA_PB_BbsI_PGKpuro2ABFP (Metzakopian, Strong et al. 2017)
	Fragment3_R	**GATCTACAGCTGCCTTGTAAGTCATTGGTCTTAAAGGGTAC**TTAGGCACCGGGCTTGCGGGTC	

Methods Table 2.3 | Genotyping primers. Used for either PCR or Surveyor / TIDE analysis.

Context	Primer Name	Primer Sequence
Elf5 targeting	Elf5_Genotyping_FOR	GCTTCCACTGGATATTACTAGGAGACAC
	Elf5_Genotyping_REV	GGAAGTTAGGGTTCAGGGCACTC
Elf5 gRNA 1 Off-Targets	Elf5_g1_OffT1_NM134029_F	CTTTACCTCCTGCCACAACTACCAC
	Elf5_g1_OffT1_NM134029_R	CTTGGGAGAAGAGTAGGGACGAGG
	Elf5_g1_OffT2_NM001013766_F	CCAGATGATTGCAAACTCTTGGAAG
	Elf5_g1_OffT2_NM001013766_R	GTTTGGGACATGGCAAAGGAC
	Elf5_g1_OffT3_NM001083902_F	GCCAAGCAGTTTCTCTTTCCATTCC
	Elf5_g1_OffT3_NM001083902_R	CTTGGTGTCACCTGATCATCCTGG
Oct4 KO	Oct4_Sur_F1	GTGCAGTGCCAACAGGCTTT
	Oct4_Sur_R1	CTGGTGTACAGACAGTGATGGCA
	Oct4_Sur_F2	GACACAGGCAGATAGCGCT
	Oct4_Sur_R2	CCACACATGGCCCAAACAAG
Dnmt1 KO	Dnmt1_Sur_ex5_F	CTGGCCTCTGGCTTAGTTTCTGT
	Dnmt1_Sur_ex5_R	AATGAATGTCTATGCGAAGCCACG
	Dnmt1_Sur_ex19_F	GTACCATGCATGTGGGGTCATATAGG
	Dnmt1_Sur_ex19_R	GCCAAATGGATCAGGGCTCTCATAC
	Dnmt1_Sur_ex28_F	GGGCAGCCAGCTTTGTCATAAG
	Dnmt1_Sur_ex28_R	GCCAAGGACTGGAAAACAAACTCC
	Dnmt1_Sur_ex30_F	CCGAGACTGTTTTTGTAGGCGTC
	Dnmt1_Sur_ex30_R	GGAATCCTTCCGATAACCCTCCA
	Dnmt1_Sur_ex31_F	CAGTGTGAGGGGATCCATGCATTTC
	Dnmt1_Sur_ex31_R	CCCACAAACCTGAATCTAGACTGGG
Bcor KO	Bcor_Sur_F	GCGCTTGCTTACCTTTTCCC
	Bcor_Sur_R	GGAGATGTGTAGCAGTCGCA
Rnf2 KO	Rnf2_g1_Sur_F	CCGACAGGTAGGACACTCTTTG
	Rnf2_g1_Sur_R	GCTTACATTACAGCTGTGGTCCTG

Methods Table 2.4 | Sequences of gRNAs used in this work.

Context	Gene_gRNA#	Sense Oligo	Antisense Oligo
Elf5 targeting	Elf5_1	CACCGGGACACCAGGCTCATTTGAGT	TAAAACTCAAATGAGCCTGGTGTCCC
	Elf5_2	CACCGCTGATGGACACCGGACACCGT	TAAAACGGTGTCCGGTGTCCATCAGC
	Elf5_3	CACCGATCAAATGAGCCTGGTGTCGT	TAAAACGACACCAGGCTCATTTGATC
	Elf5_4	CACCGGAGAAACTCTGATGGACACGT	TAAAACGTGTCCATCAGAGTTTCTCC
Oct4 KO	Oct4_1	CACCGACTCGTATGCGGGCGGACAGT	TAAAACTGTCCGCCCGCATACGAGTC
	Oct4_7	CACCGCCGCATACGAGTTCTGCGGGT	TAAAACCCGCAGAACTCGTATGCGGC
	Oct4_10	CACCGCTGAGCCTGGTCCGATTCCGT	TAAAACGGAATCGGACCAGGCTCAGC
	Oct4_11	CACCGCTAGTCCCCCAAGTTGGCGGT	TAAAACCGCCAACTTGGGGGACTAGC
	Oct4_13	CACCGTTCACGGCATTGGGGCGGTGT	TAAAACACCGCCCCAATGCCGTGAAC
	Oct4_15	CACCGCATTGGGGCGGTCGGCACAGT	TAAAACTGTGCCGACCGCCCCAATGC
Dnmt1 KO	Dnmt1_1_ex19	CACCGGACCTGATCAATAAGATTGGT	TAAAACCAATCTTATTGATCAGGTCC
	Dnmt1_2_ex19	CACCGTTGGCTCATACTCTTTGCTGT	TAAAACAGCAAAGAGTATGAGCCAAC
	Dnmt1_3_ex28	CACCGTTATCCGACCGATGCGATAGT	TAAAACTATCGCATCGGTCGGATAAC
	Dnmt1_4_ex28	CACCGATACTTGCGGTAGTGCTCAGT	TAAAACTGAGCACTACCGCAAGTATC
	Dnmt1_5_ex30	CACCGCCGCAGCCCTGGGAACAAAGT	TAAAACTTTGTTCCCAGGGCTGCGGC
	Dnmt1_6_ex30	CACCGGGTGGGTCTTCAAAGTTCTGT	TAAAACAGAACTTTGAAGACCCACCC
	Dnmt1_7_ex31	CACCGCAGCCGGAAAACACATCCAGT	TAAAACTGGATGTGTTTTCCGGCTGC
	Dnmt1_8_ex31	CACCGGCTGTGGAGGGTTATCGGAGT	TAAAACTCCGATAACCCTCCACAGCC
	Dnmt1_9_ex5	CACCGGAAACTTCACCTAGTTCCGGT	TAAAACCGGAACTAGGTGAAGTTTCC
	Dnmt1_10_ex5	CACCGTTCGTGAAGTGAGCCGTGAGT	TAAAACTCACGGCTCACTTCACGAAC
Screening Validation	Non-Targeting_1	CACCGGTACTGGACATTCTTATCCGT	TTAAACGGATAAGAATGTCCAGTACC
	Non-Targeting_2	CACCGTCCAGCTTATGATTGGCGCGT	TTAAACGCGCCAATCATAAGCTGGAC
	1_p53 _1	CACCGAAGTCACAGCACATGACGGGT	TTAAACCCGTCATGTGCTGTGACTTC
	1_p53 _2	CACCGTCGGAGCAGCGCTCATGGTGT	TTAAACACCATGAGCGCTGCTCCGAC
	2_Tsc1 _1	CACCGTGCAATACCGGCTGAGAATGT	TTAAACATTCTCAGCCGGTATTGCAC
	2_Tsc1 _2	CACCGCCTATGCTTGTCAACACGTGT	TTAAACACGTGTTGACAAGCATAGGC
	3_Rock2 _1	CACCGGCTGAAGTAGTGCTTGCACGT	TTAAACGTGCAAGCACTACTTCAGCC
	3_Rock2 _2	CACCGTGATGGAGTACATGCCAGGGT	TTAAACCCTGGCATGTACTCCATCAC
	4_Mcoln1 _1	CACCGGTGACAAGTTCCGGGCCAAGT	TTAAACTTGGCCCGGAACTTGTCACC
	4_Mcoln1 _2	CACCGCAGGTGGTCAAGATCTTGGGT	TTAAACCCAAGATCTTGACCACCTGC
	5_Nf2 _1	CACCGTTTGGTGTGCCGGACACTGGT	TTAAACCAGTGTCCGGCACACCAAAC
	5_Nf2 _2	CACCGGATTTGGTGTGCCGGACACGT	TTAAACGTGTCCGGCACACCAAATCC
	6_Tsc2 _1	CACCGACAATCGCATCCGAATGATGT	TTAAACATCATTCGGATGCGATTGTC
	6_Tsc2 _2	CACCGCATTCGGATGCGATTGTTGGT	TTAAACCAACAATCGCATCCGAATGC
	7_Pten _1	CACCGATCCCATAGCAATAATATTGT	TTAAACAATATTATTGCTATGGGATC

7_Pten _2	CACCGAAGCTGGAAAGGGACGGACGT	TTAAACGTCCGTCCCTTTCCAGCTTC
8_Bcor _1	CACCGGGAGTCTTTGGTTGCTGGGGT	TTAAACCCCAGCAACCAAAGACTCCC
8_Bcor _2	CACCGTTGCACACCTCGGAGTCTTGT	TTAAACAAGACTCCGAGGTGTGCAAC
9_Hira _1	CACCGATGTGTGAACTGTGTGCGGGT	TTAAACCCGCACACAGTTCACACATC
9_Hira _2	CACCGGGAGATGACAAACTGATTAGT	TTAAACTAATCAGTTTGTCATCTCCC
10_Lats1 _1	CACCGAGAGACACGGCCCATCTCTGT	TTAAACAGAGATGGGCCGTGTCTCTC
10_Lats1 _2	CACCGTCTGAAAGCCCCAACTCACGT	TTAAACGTGAGTTGGGGCTTTCAGAC
11_Asxl1 _1	CACCGGCATTGAGGCACGCAAGAGGT	TTAAACCTCTTGCGTGCCTCAATGCC
11_Asxl1 _2	CACCGTGGCATTGAGGCACGCAAGGT	TTAAACCTTGCGTGCCTCAATGCCAC
12_Vps16 _1	CACCGGAGGAGCTCAAGGATTGCCGT	TTAAACGGCAATCCTTGAGCTCCTCC
12_Vps16 _2	CACCGGTGGCTGCTGCACCCTATGGT	TTAAACCATAGGGTGCAGCAGCCACC
13_Kctd5 _1	CACCGGGATCTCTGTCGATTAAATGT	TTAAACATTTAATCGACAGAGATCCC
13_Kctd5 _2	CACCGATTAACAAAGACCTCGCAGGT	TTAAACCTGCGAGGTCTTTGTTAATC
14_Smad2 _1	CACCGTCTCTTGATGGCCGTCTTCGT	TTAAACGAAGACGGCCATCAAGAGAC
14_Smad2 _2	CACCGGGTCTCAACTCTCTGGTAGGT	TTAAACCTACCAGAGAGTTGAGACCC
15_Spns1 _1	CACCGAGTTCTTTAACATCGGAGAGT	TTAAACTCTCCGATGTTAAAGAACTC
15_Spns1 _2	CACCGACATCGGAGATGGTAGTACGT	TTAAACGTACTACCATCTCCGATGTC
16_Socs3 _1	CACCGGATCCAGGAACTCCCGAATGT	TTAAACATTCGGGAGTTCCTGGATCC
16_Socs3 _2	CACCGGAACCCTCGTCCGAAGTTCGT	TTAAACGAACTTCGGACGAGGGTTCC
17_Tet1 _1	CACCGCATCCTTCTCCGGCTTGGAGT	TTAAACTCCAAGCCGGAGAAGGATGC
17_Tet1 _2	CACCGCCATGGACTGCAGTAGACGGT	TTAAACCGTCTACTGCAGTCCATGGC
18 _Fubp1 _1	CACCGTCAAATGACTATGGTTATGGT	TTAAACCATAACCATAGTCATTTGAC
18 _Fubp1 _2	CACCGATTCAAATGACTATGGTTAGT	TTAAACTAACCATAGTCATTTGAATC
19_Rnf2 _1	CACCGTTAATGTGCCCAATTTGTTGT	TTAAACAACAAATTGGGCACATTAAC
19_Rnf2 _2	CACCGTGTTTACATCGGTTTTGCGGT	TTAAACCGCAAAACCGATGTAAACAC
20_Cnot8 _1	CACCGGTGTTGTTGTACGGCCGATGT	TTAAACATCGGCCGTACAACAACACC
20_Cnot8 _2	CACCGCCCGTCTGGAATCAACACAGT	TTAAACTGTGTTGATTCCAGACGGGC
21_Ccdc101 _1	CACCGTCTGTCTGCATCCGCTCGTGT	TTAAACACGAGCGGATGCAGACAGAC
21_Ccdc101 _2	CACCGCATCCAGAAAACCCACGAGGT	TTAAACCTCGTGGGTTTTCTGGATGC
22_Rybp _1_K5-3	CACCGAATATCAGACTTTGGTCTAGT	TTAAACTAGACCAAAGTCTGATATTC
22_Rybp _2_K5-4	CACCGCCAGCTGAGAATTGATGCGGT	TTAAACCGCATCAATTCTCAGCTGGC
23_Taf5l _1	CACCGAAACTGCACTTCATACTGCGT	TTAAACGCAGTATGAAGTGCAGTTTC
23_Taf5l _2	CACCGGAATCTGGTTGTGCCAATGGT	TTAAACCATTGGCACAACCAGATTCC
24_Rprd2 _1	CACCGAAAAGAGATTCAAACGATGGT	TTAAACCATCGTTTGAATCTCTTTTC
24_Rprd2 _2	CACCGATAAAAGAGATTCAAACGAGT	TTAAACTCGTTTGAATCTCTTTTATC
25_Spop _1	CACCGAACTTTAGTTTTTGCCGGGGT	TTAAACCCCGGCAAAAACTAAAGTTC
25_Spop _2	CACCGTTATTGATGGTCCACATGTGT	TTAAACACATGTGGACCATCAATAAC

	26_Slc11a2 _1	CACCGGGCAGGGTTGATGGCGCCAGT	TTAAACTGGCGCCATCAACCCTGCCC
	26_Slc11a2 _2	CACCGGCATTGCCTACCTAGACCCGT	TTAAACGGGTCTAGGTAGGCAATGCC
	27_Lipt2 _1	CACCGCTTTGAAGCGCCTCACTCAGT	TTAAACTGAGTGAGGCGCTTCAAAGC
	27_Lipt2 _2	CACCGCTGTTCAACCGACCTCACAGT	TTAAACTGTGAGGTCGGTTGAACAGC
	28_Trim24 _1	CACCGACTTTTCTACTGTACTACTGT	TTAAACAGTAGTACAGTAGAAAAGTC
	28_Trim24 _2	CACCGACACTCTTGACTGCAAACTGT	TTAAACAGTTTGCAGTCAAGAGTGTC
	29_Stk35 _1	CACCGAGTGCGTCCTACAGCGCAAGT	TTAAACTTGCGCTGTAGGACGCACTC
	29_Stk35 _2	CACCGAGCTACGGCGTGGTTTATGGT	TTAAACCATAAACCACGCCGTAGCTC
	30_Supt20 _1	CACCGAAAGGAAATACCTGTCTAGGT	TTAAACCTAGACAGGTATTTCCTTTC
	30_Supt20 _2	CACCGTCAGCAGAGACCTCCTAAAGT	TTAAACTTTAGGAGGTCTCTGCTGAC
	31_Lipt1 _1	CACCGTCGGGTGCCTTAATAGCGCGT	TTAAACGCGCTATTAAGGCACCCGAC
	31_Lipt1 _2	CACCGCCGTATGAGCAGGCGCATTGT	TTAAACAATGCGCCTGCTCATACGGC
	32_Pcgf1 _1	CACCGTATGGCTGTCCCGCTGGTTGT	TTAAACAACCAGCGGGACAGCCATAC
	32_Pcgf1 _2	CACCGGAGAACCTCATTGTCAAAAGT	TTAAACTTTTGACAATGAGGTTCTCC
	33_Taf6l _1	CACCGGGTGATCTCTACTTCCCGGGT	TTAAACCCGGGAAGTAGAGATCACCC
	33_Taf6l _2	CACCGGCTCACTTCTCGATCCTCCGT	TTAAACGGAGGATCGAGAAGTGAGCC
	34_Cnfn _1	CACCGGCCGCATCTCCGATGACTTGT	TTAAACAAGTCATCGGAGATGCGGCC
	34_Cnfn _2	CACCGGCAGACCTCCGGGCAGGTAGT	TTAAACTACCTGCCCGGAGGTCTGCC
	35_Psmf1 _1	CACCGTATTGCTGTTCCACTTAGCGT	TTAAACGCTAAGTGGAACAGCAATAC
	35_Psmf1 _2	CACCGTCCATCCTTAGACTCATACGT	TTAAACGTATGAGTCTAAGGATGGAC

Methods Table 2.5 | List of mouse Taqman Probes for qRT-PCR.

Gene Name	Applied Biosystems Assay ID
Ascl2	Mm01268891_g1
Bcor	Mm01291502_m1
Cdx2	Mm01212280_m1
Dio3	Mm00439576_m1
Dnmt1	Mm01151063_m1
Elf5	Mm00468732_m1
Eomes	Mm01351984_m1
Esrrb	Mm00442411_m1
Fgf5	Mm00438615_m1
FgrR2	Mm01269930_m1
Gadph	4352339E
Gata3	Mm00484683_m1
Gata6	Mm00802636_m1
Gcm1	Mm00492310_m1
Gm52 (Syncytin a)	Mm02744887_s1
H19	Mm01156721_g1
Hand1	Mm00433931_m1
Igf2R	Mm00439576_m1
IntA7	Mm00434400_m1
Klf4	Mm00516104_m1
Nanog	Mm02384862_g1
Pcgf1	Mm01617932_g1
Peg10	Mm01167724_m1
Peg3	Mm01337379_m1
Pou5f1 (Oct4)	Mm00658129_gH
Prl2c4 (Prl2c2, Plf)	Mm04208104_gH
Prl3b1 (PL2)	Mm00435852_m1
Prl3d1 (PL1)	Mm04213281_g1
Rnf2	Mm00803321_m1
Rybp	Mm00451094_m1
Sox1	Mm00486299_s1
Sox2	Mm03053810_s1
Syncitin b	Mm04212068_g1
T	Mm01318252_m1
Tfap2a	Mm00495574_m1
Tfap2c	Mm01352548_g1
Tpbpa	Mm00493788_g1
Zfp42 (Rex1)	Mm03053975_g1

Methods Table 2.6 | Primers used for *Elf5* promoter DMR analysis.

Primer Name	Primer Sequence
Elf5_DMR1_MH_F	TTTGTAGTTTGAGTATTTTGGTG
Elf5_DMR1_MH_R	ACCTTTCCACTCTAAACACCCAAA
Elf5_DMR2_F	GTTTTTTGGGTGTTTAGAGT
Elf5_DMR2_R	CATTACAACCTTTACCTAAACTA

Methods Table 2.7 | List of antibodies.

Application	Antibody	Company	Catalogue Number	Dilution
Western Blot	Rnf2	Abcam	ab181140	1:1000
	TY1	Diagenode	C15200054	1:1000
	Histone H3	Abcam	ab1791	1:1000
	Lamin B	Santa-Cruz	sc-374015	1:500
	Goat anti-mouse IgG-HRP	Santa-Cruz	sc-2005	1:5000
	Goat anti-rabbit IgG-HRP	Invitrogen	A16116	1:10.000
Immuno-cytochemistry And Embryo staining	Oct4	Abcam	ab181557 ab19857	1:250 1:250
	Nanog	Abcam	ab80892	1:250
	Cdx2	Biogenex	MU392A-UC	1:150
	GFP	Abcam	ab13970	1:1000
	Elf5	Santa-Cruz	sc-9645	1:50
	Gata3	Santa-Cruz	sc-1236	1:150
	PL1	Santa-Cruz	sc-34713	1:50
	mCherry	Abcam	ab167453	1:200
	Gata6	Santa-Cruz	sc-7244	1:50
	Goat anti-mouse AF488	Invitrogen	A11029	1:500 – 1:1000
	Goat anti-mouse AF594	Invitrogen	A11005	1:500 – 1:1000
	Goat anti-Chicken AF488	Abcam	ab150169	1:500
	Goat anti-Rabbit CF647	Biotium	20043-1-BT	1:500
	Goat anti-Rabbit AF488	Invitrogen	A11008	1:1000
	Goat anti-Rabbit AF594	Invitrogen	A11037	1:1000
	Donkey anti-Goat AF594	Invitrogen	A11058	1:1000
Flow Cytometry	Oct4	Abcam	ab19857	1:100
	Cdx2	Biogenex	MU392A-UC	1:100
	Dnmt1	Abcam	ab87656	1:500
	Integrin alpha-7	Bio-techne (R&D Systems)	FAB3518P	1:10
	Goat anti-rabbit AF594	Invitrogen	A11012	1:2000
	Goat anti-mouse AF594	Invitrogen	A11005	1:2000