Chapter 2

Materials and Methods

2.1 Cell Culture

2.1.1 Materials

2.1.1.1 Cell lines

Cells	Source
Rex1:GFPd2 reporter cell line	Mouse E14 with expression of a destabilised GFP (GFPd2) driven by the endogenous *Rex1* promoter [450]
Cas9-expressing JM8 cell line	Generated by Koike-Yusa et al. [202]
HEK293FT	Human embryonic kidney cells obtained from Invitrogen

2.1.1.2 Media components, inhibitors and other reagents used in cell culture

Components	Supplier	Catalog No.
Knockout DMEM	Life Technologies	10829018
DMEM	Life Technologies	21969
Fetal Bovine Serum (FBS)	Life Technologies	10500064
GlutaMax (100X)	Life Technologies	35050061
Non-essential Amino Acids (100X)	Life Technologies	11140035
β-Mercaptoethanol	Sigma-Aldrich	M6250
NDiff 227	Clontech	Y40002
Knockout Serum Replacement (KSR)	Life Technologies	10828
20% Bovine Serum Albumin (BSA)	Life Technologies	15260037
Penicillian-Streptomycin (100X)	Life Technologies	5140122
PD0325901	Selleck Chemicals	S1036
CHIR99021	Selleck Chemicals	S1263
Leukemia inhibitory factor (LIF)	Millipore	ESG1107
G418, Geneticin	Invitrogen	10131027
Rapamycin	Sigma Aldrich	R8781
Dimethyl Sulfoxide (DMSO)	Sigma Aldrich	276855
Phosphate Buffered Saline (PBS)	Sigma-Aldrich	806544

2.1.1.3 Dissociation agents

Components	Supplier	Catalog No.
Trypsin- 0.25% EDTA	Life Technologies	25200056
Accutase	Millipore	SCR005

2.1.1.4 Other chemicals and kits

Components	Supplier	Catalog No.
Leukocyte Alkaline Phosphatase kit	Sigma-Aldrich	86R-1KT
Paraformaldehyde (PFA)	Sigma-Aldrich	158127
Mytomycin C	Sigma-Aldrich	50-07-7
Polybrene	Sigma-Aldrich	86R-1KT
Lipofectamin LTX	Invitrogen	15338100

2.1.1.5 Media

Media	Components
Serum/LIF condition	KnockOut DMEM supplemented with: 15%FBS 1 x GlutaMax 1 x NEAA 0.1mM β-mercaptoethanol 1000 U/mL LIF
2i/LIF condition	NDiff 227 supplemented with: 5%BSA 1%KSR 1 x NEAA 0.1mM β-mercaptoethanol 1 μM PD0325901 3 μM CHIR99021 1000 U/mL LIF
Medium for feeder cells	KnockOut DMEM supplemented with: 10%FBS 1 x GlutaMax 1 x NEAA 0.1mM β-mercaptoethanol
Medium for HEK293FT (D10)	DMEM supplemented with: 10%FBS 1 x GlutaMax

2.1.2 Methods

2.1.2.1 Routine culture and maintenance mESCs

ESCs cultured under serum/LIF condition were routinely passaged on mitotic-inactivated feeder layer everyday at a split ratio of 1:2-1:3. Trypsin-0.25% EDTA was used followed by mechanistic dissociation to achieve single cell suspension before replating. Feeder cells were prepared by treating MEF with 15 µg/mL mitomycin C for 3 hours. Feeder cells were plated one day before passaging.
ESCs cultured in 2i/LIF were passaged every two or three days on 0.1% gelatin coated plastics at a split ratio of 1:8-1:10 dependng on confluency. Accutase was used as a dissociation agent. Medium was replenished everyday. For freezing, mESCs were harvested and resuspended in freezing medium (90% culture medium, 10%DMSO) and stored overning at -80 ° C before transfer into liquid nitrogen for long-term storage.

2.1.2.2 Differentiation condition

ESCs were detached with Accutase and plated at a density of 10,000/cm^2 in N2B27 medium on 0.1% gelatin coated plates in the absence of LIF or 2i to allow differentiation. Medium was changed every day.

2.1.2.3 Transfection of mESCs

Lipofection

Lipofection was conducted using the Lipofectamine LTX Reagent as per manufacturer's protocol. For 4 x 10^5 ESCs, the DNA transfection complex was prepared as follows: 2 µg kit-purified DNA and 2 µPLUS reagent were mixed with 500 µL OptiMEM and incubated at room temperature for 5 minutes. LipofectamineLTX reagent (6 µL) was added and further incubated for 30 minutes. The DNA transfection complex was mixed with cell suspension and plated on MEF in serum/LIF. Medium was replenished after 3 hours.

Electroporation

A suspension of 10 x 10^7 ESCs were mixed with 25 µg DNA in 800 µL PBS. The mixture was transferred to a 0.4 cm gap cuvette (Biorad) and electroporated at 240 V, 500 µF using the Gene Pulser Xcell Electroporation systems (Biorad). The electroporated cells were plated on feeders in serum/LIF at a density of approximately 50,000 cells per cm^2.

Selective drugs were added about 16 to18 hours later. Medium was replenished every day. Colonies were picked and genotyped about 6 to 8 days later.

2.1.2.4 Lentivirus production and transduction

Lentifivus production

HEK293FT cells were plated the day before transfection at a density to achieve 70-80% confluency at the time of transfection. To produce virus from one 10-cm cell culture dish, 5.4 μg lentiviral transfer vector, 5.4 μg psPax2, 1.2 μg pMG2.G and 12 μL PLUS reagent were mixed in 3 mL Opti-MEM and incubated for 5 minutes at room temperature. 36 μL Lipofectamine LTX was added to the mixture and further incubated for 30 minutes at room temperature. At the end of the incubation, D10 was replaced with 5 mL OptiMEM from the dish culturing HEK293FT cells before DNA/Lipofectamine complex was added to the cells. The cells were incubated with the DNA/Lipofectamine complex for 6 - 8 hours, after which medium was replaced with 10 mL D10. The amount of reagents were scaled up or down accordingly depending on the amount of virus to be produced. Two days after transfection, viral supernatant was collected with a syringe and filtered through a 0.45 μm filter cartridge. The filtered viral supernatant was aliquoted and stored at -80 ° C.

Lentivirus transduction

0.5 x 10^6 ESCs were resuspended in 500 μL diluted viral supernatant supplemented with polybrene at a final concentration of 4 μg/mL. The ESC-lentivirus suspension was incubated at 37 ° C for 30 minutes before being plated in serum/LIF on feeders. Medium was changed the next day. The amount of reagents was scaled up or down depending on the amount of cells to be transduced.

2.1.2.5 Genetic manipulation of cell lines

Cas9 knockin

The *Rosa26* Cas9 targeting vector (Figure 3.2) was linearised with PacI and introduced into the Rex:GFPd2 cells by electroporation. Transfected cells were selected in 180 mM G418 for 8 days, after which single colonies were picked and expanded. Genomic PCR was performed using LongAmp Taq DNA polymerase (NEB) with the following primers:
Forward primer: TCGCATTGTCTGAGTAGGTGTCATTCTA
Reverse primer: CTAACAAAACGTCTCAACTTCAAGGTGA

Generation of stable knockout cell lines

Several stable knockout mESC lines were generated using paired gRNAs to delete one ′critical′ exon, which is defined as an exon that is common to all transcripts and creates a frame-shift mutation when deleted.. Cas9-expressing Rex1GFPd2 cells were transfected with 2 plasmids encoding gRNA#1 (1 μg) , gRNA#2 (1 μg). gRNA sequence as shown in Table:2.1. Three days after transfection, BFP-positive cells were sorted and plated in serum/LIF on MEF at a density of 1000 cells per 10cm dish. Single colonies were picked and genotyped after 6 to 8 days. PCR genotyping primer sequence as shown in Table:2.2.

Table 2.1: gRNA pairs used to generate stable knockout cell lines

Gene	gRNA1	gRNA2
Tcf7l1	GCTCCCAAAGAGCGGTGGTG	TGAAAGGAGCCACCGGTGAG
Apc	ACAAGCTAATACATATTGCC	GACAGTGCAGCTTTTAGATT
Tsc1	GGCGACATCAGGCTCAGCAC	GCAGCCATGTGTATGCGGGA
Tsc2	AAAAGGTGCTGCAGTTCACG	TCCAAGCTTAATGCATTAGG
Nprl2	CCGGACCCAACGTCCACTGA	GTGTGAGGCTTTAGTTGGGT
Depdc5	CACAGGGCACCCCATCATGT	ACAAAACATGCTCGTCTCTA
Rraga	GTCGCCACGGACTGGGCCTG	CTGATAGACGATGCTGGACC
Rictor	TGCTTGTCTATGCACAATTT	TAACAATTTAAGTCCGAGCT

Table 2.2: PCR genotyping primers

Gene	Forward primer	Reverse primer
Tcf7l1	AGCCATTTTGACGTCTGTCC	CCGAGAGCTCCTGTCAGAAC
Apc	CGTCAGTGCAGTGTTCCTTC	AGTCTGAAGTCAGCCCAGGA
Tsc1	GGGGATAGGGATAGGGGTCT	ATGAACTGCAGGGTTTCTGG
Tsc2	GACAGGAGGCAAGCAGAAAC	GCTAGAGAAGGGCAGGGAGT
Nprl2	CAAAGTAGACCACTGGGTGGA	AGAAGAAGCTGATTGGCTGC
Depdc5	ACTCTCAGGGAAAAGGCAGA	TGCTTTTGCAAGTCAAGTCG
Rraga	TGGTCTGCTCTCGCTAGCTC	TTTGCCCAGATTATTGAGGC
Rictor	ACGGTGGGACAGAAACTCAG	TCAAGCAGTTTCAGTGCCAC

2.1.2.6 Commitment assay

ESCs were plated under differentiation condition for 26-28 hours before being detached and replated in N2B27 supplemented 2i/LIF at a density of approximately 20,000 cells per well of a 12-well plate. Uncommitted ESCs were allowed to expand for 6-8 days before AP staining was performed. Medium was replenished every day.

2.1.2.7 Flow cytometry and cell sorting

Flow cytometric analysis was performed to analyse Rex1-GFP expression. ESCs were dissociated into single cells by trypsin and resuspended in 1% (V/V) BSA in PBS followed by filtration through a 35 μm cell strainer to remove any cell clumps. Filtered cells were immediately brought for analysis using the LSR Fortessa instrument (Becton Dickinson). Data was analysed using FlowJo.

For transduction efficiency measurements, harvested ESCs were fixed with 4% (V/V) PFA for 20 minutes. The fixed cells were washed twice with PBS before being resuspended in 1% BSA and analysed on the LSR Fortessa instrument.

To perform fluorescent activated cell sorting (FACS), harvested ESCs were filtered and resuspended in culture medium to a final density of approximately 5 x 10^6 per ml and left on ice until sorting. The sorted cells were either pelleted down and stored at -80° C for DNA/RNA extraction, or plated in culture medium supplement with Penicillin-Streptomycin for further analysis.

2.2 Molecular Biology

2.2.1 Materials

2.2.1.1 Molecular chemicals and Kits

Reagents	Supplier	Catalog No.
1 kb DNA ladder	NEB	N3232S
100 bp DNA ladder	NEB	N3231S
Ampicillin	Sigma Aldrich	59349
DH5α competent cells	Invitrogen	18263012
LongAmp Taq DNA polymerase	NEB	M0323S
Q5 Hot Start High-Fidelity 2XMaster Mix	NEB	M0494S
10x Ligation buffer	NEB	M0202S
T4 Ligase	NEB	M0202S
T4 PNK	NEB	M0201
Proteinase K	Roche	03115879001
DNeasy Blood and Tissue Kit	Qiagen	69504
Blood and Cell Culture DNA Midi Kit	Qiagen	13343
Blood and Cell Culture DNA Maxi Kit	Qiagen	13362
QIAprep Spin Miniprep Kit	Qiagen	27104
QIAquick PCR Purification Kit	Qiagen	28104
RNeasy Mini Kit	Qiagen	74104
Agencourt AMPure XP beads	Beckman	A63881
DynaMag-96 Side Magnet	Invitrogen	12331D

2.2.1.2 Immunoblotting

Reagents	Supplier	Catalog No.
RIPA buffer	Sigma-Aldrich	R0268
Protease Inhibitor Cocktail	Sigma-Aldrich	P8340
Phophatase Inhibitor Cocktail	Sigma-Aldrich	P5726
Bradford protein assay	Bio-Rad	5000006
Colour Prestained Protein Standard (11-245 kDa)	NEB	P7712
NuPAGE MOPS SDS Running Buffer (20X)	Invitrogen	NP0001
NuPAGE Transfer Buffer (20X)	Invitrogen	NP00061
BSA lyophilized powder	Sigma-Aldrich	A9647
Nonfat dry milk blotting-grade blocker	Bio-Rad	1706404
NuPAGE Sample Reducing Agent (10X)	Invitrogen	NP0009
NuPAGE LDS Sample Buffer (4X)	Invitrogen	NP0007
NuPAGE Novex 4-12% Bis-Tris Protein Gels, 10-well	Invitrogen	NP0321
NuPAGE Novex 4-12% Bis-Tris Protein Gels, 12-well	Invitrogen	NP0322
Pierce TBS Buffer (20X)	Thermo Scientific	28358
Tween 20	Sigma-Aldrich	90050-64-5
Thick Blot Paper	Bio-Rad	1703932
Amersham ECL start Wester Blotting Detection Reagent	GE Healthcare	RPN3244
Amersham Hypercassette	GE Healthcare	RPN11642
Amersham Hyperfilm ECL	GE Healthcare	28906837
Amersham Hybond P 0.45 PVDF	GE Healthcare	10600023
Cell scraper	Corning	734-1537
Mini Trans-Blot Electrophoretic Transfer Cell	Bio-Rad	1703930
XCekk SureLock Mini-Cell Electrophoresis System	Thermo Fisher	EI0002

Antigen	Supplier	Source	Dilution
Akt-pThr308	Cell Signaling-9275	Rabbit/Polyclonal	1:1000
Akt-pSer473	Cell Signaling-9271	Rabbit/Polyclonal	1:1000
Akt	Cell Signaling-9272	Rabbit/Polyclonal	1:1000
S6K-pThr389	Cell Signaling-9205	Rabbit/Polyclonal	1:1000
S6K	Cell Signaling-9202	Rabbit/Polyclonal	1:1000
S6-pSer235/236	Cell Signaling-2211	Rabbit/Polyclonal	1:1000
S6	Cell Signaling-2217	Rabbit/Monoclonal	1:1000
GSK3β-pSer9	Cell Signaling-5558	Rabbit/Monoclonal	1:1000
GSK3β	Santa Cruz-9166	Rabbit/Polyclonal	1:1000
PRAS40-pThr246	Cell Signaling-13175	Rabbit/Monoclonal	1:1000
PRAS40	Cell Signaling-2691	Rabbit/Monoclonal	1:1000
β-Actin	Sigma-Aldrich-A2228	Mouse/Monoclonal	1:1000
Rabbit IgG-HRP	GE Healthcare-NA934	Donkey	1:1000
Mouse IgG-HRP	GE Healthcare-NA931	Donkey	1:1000

2.2.2 Methods

2.2.2.1 Isolation of nucleic acids

Plasmid DNA was obtained using the QIAprep Spin Miniprep Kit. Genomic DNA was obtained from mESCs collected from cell sorting or direct trypsinisation. DNeasy Blood and Tissue kit was used when cell number was less than 5 million. Blood and Cell Culture DNA Midi kit was used when cell number was in between 5 million and 20 million. Blood and Cell Culture DNA Maxi kit was used when cell number was above 20 million. RNA was extracted using the RNeasy Mini Kit.

2.2.2.2 gRNA cloning

gRNA expression vector (Figure 4.1 (A)) was linearised with BbsI and purified from gel. 1 pmol each of top and bottom strand oligo were mixed with 1 µL of 10X T4 ligation buffer, 0.5 µL of T4 PNK and dH_2O to achieve a total volume of 10 µL. Reaction was performed in a thermal cycler with the following conditions: 37 ° C for 30 minutes followed by 95 ° C for 5 minutes, after which temperature was set to ramp down to 25 ° C at 0.1 ° C/second. 14.2 fmol annealed ds-oligo was ligated with 3.7 fmol linearised vector by mixing with 1 µL T4 ligase and 1 µL 10X ligase buffer in a total volume of 10 µL. The ligation mixture was incubated at 16 ° C for 4-16 hours. Ligated plasmid was introduced into DH5 chemical competent cells following standard bacteria transformation protocol. Bulk plasmid was prepared by inoculating 2 mL of 2X YT media (50 µg/mL Amp) and shaking overnight at 37 ° C. Plasmid DNA was purified with Miniprep kit. To prepare single clone, transformed bacteria was plated on LB Amp plates followed by expanding in liquid culture. Plasmid sequence was checked by capillary sequencing.

2.2.2.3 Lysate PCR

Approximately 5,000 cells were collected per PCR tube and washed twice with PBS. 25 µL water was added to the cell pellet and heated for 10 minutes at 90 ° C for cell lysis. 5 µL proteinase K was added to each well and reaction was carried out at 55 ° C for 60 minutes. The enzyme was heat inactivated at 95 ° C for 10 minutes. PCR was carried out using 10 µL cell lysate in a 50 µL PCR reaction with LongAmp Taq DNA polymerase following the manufacturer's protocol.

2.2.2.4 Illumina library preparation

At the end of the screen, gRNAs were amplified from lentivirally transduced cells using the following primers:
Forward primer: ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGTGGAAAG-GACGAAACA; Reverse primer: TCGGCATTCCTGCTGAACCGCTCTTCCGATCTC-TAAAGCGCATGCTCCAGAC. The following reaction mix was set up:

Reagent	Volume per reaction
2x Q5 HS HF	25 µL
Primer mix (10 µM each)	1 µL
Genomic DNA	2 µg
dH_2O	up to 50 µL

To cover the complexity of the gRNA library, approximately 72 µg genomic DNA was analysed for the control samples. All the genomic DNA was analysed for sorted Rex1-GFP positive cells. The PCR reaction was set to run with the following programme:

Cycle number	Denature	Annealing	Extension
1	98 ° C, 30 seconds		
2-26	98 ° C, 30 seconds	61 ° C, 15 seconds	72 ° C, 20 seconds
27			72 ° C, 2 minutes

The PCR end product was purified using QIagen's PCR purification kit and used for second round PCR.

Reagent	Volume per reaction
2x KAPA HiFi HotStart ReadyMix	25 µL
Primer mix (5 µM each)	2 µL
First round PCR product	1 ng
dH_2O	18 µL

Cycle number	Denature	Annealing	Extension
1	98 ° C, 30 seconds		
2-9	98 ° C, 10 seconds	66 ° C, 15 seconds	72 ° C, 20 seconds
10			72 ° C, 5 minutes

The end PCR product was purified using SPRI beads and transferred for Illumina sequencing.

2.2.2.5 Western blotting

ESCs were lysed on tissue culture plate directly by adding RIPA buffer supplemented with phosphatase inhibitor (1:100) and protease inhibitor (1:1000). Protein was purified from cell debris via centrifugation at 15000 rpm for 15 minutes at 4 ° C. Total protein content was quantified using Bradford Protein Assay (BioRad). Protein standard was prepared by diluting BSA to a range of 0.05 to 0.25 mg/mL. 10 µL protein standard and sample solution was added into duplicate wells of a 96-well clear flat-bottom plate. 200 µL Bradford protein assay dye reagent was added into each well. The mixture was incubated for at least 5 minutes before measuring the absorbance at 595 nm. The protein concentration was calculated based on the standard curve obtained from the readings of protein standards. The quantified protein samples were diluted with 4X sample buffer supplemented with reducing agent before being heat denaturation. The denatured protein samples were resolved at 180 V for 1 hour through a 4-12% Bis-Tris gel in SDS running buffer, then subsequently being transferred onto a PVDF membrane at 90 V for 1 hour at 4 ° C using the wet transfer method. Blots were blocked for 30 minutes in either 5% BSA or milk in TBS buffer plus 0.1% Tween 20 (TBS-T). Blots were probed in diluted primary antibody overnight at 4 ° C followed by three times washing in with TBS-T. Membranes were then probed with appropriate horse radish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. Membranes were washed three times in TBS-T before the addition of ECL substrate and exposure onto the X-ray film.

2.3 Bioinformatics analysis

2.3.0.1 RNA-Seq analysis

The extracted RNA was sequenced on Illumina HiSeq2500 by 75-bp paired-ended sequencing. Sequencing data was analysed using Kallisto with the mouse RefSeq transcriptome as a reference [36]. Differential gene expression analysis was performed using DESeq2.

2.3.0.2 gRNA sequencing result analysis

The number of reads for each gRNA was counted with an in house scripted written by Yilong Li. The enrichment and depletion of gRNAs and genes were analysed by MAGeCK [225].

2.3.0.3 Gene Set Enrichment Analysis (GSEA) analysis

The GSEA analysis was performed using the online algorithm 'GSEAPreranked' developed and maintained by the Broad Institute (http://software.broadinstitute.org/cancer/software/genepattern/modules/docs/GSEAPreranked/1) [395]. Screening hits were pre-ranked according to the Depletion/Enrichment (DE) score, which was computed as: log_{10} (Depletion P value) + [- log_{10} (Enrichment P value)]. The 'Mitochondrion morphogenesis' and 'Oxidative phosphorylation' gene sets were downloaded from the Molecular Signatures Database (MSigDB). The 'Betschinger' gene set contains all the validated genes from a published siRNA-based screen [19].