Chapter 7

Materials and Methods

7.1 General molecular biology

7.1.1 Agarose gel electrophoresis

Agarose powder was dissolved in TAE (generally at 1% i.e. 1 g of agarose/100 ml buffer). Ethidium bromide was added (5 µl/100 ml) by heating and the gel was poured into a mould with a gel comb. When set, the gel was transferred to an electrophoresis tank and covered with TAE. Samples were mixed with loading dye and loaded into each well, with a molecular weight marker run in parallel (HyperLadder 1 kb (#BIO-33053, Bioline)). Samples were run at ~120V for 20-45 minutes and visualised using a Gel Doc XR+ System (Bio-Rad).

For instances when the DNA was to be extracted from the gel, ethidium bromide was not used. Post-electrophoresis staining was carried out using SYBR™ Green I Nucleic Acid Gel Stain (#S7563, Invitrogen) diluted in TAE; gels were incubated at room temperature for 30 minutes in darkness. An LED-based trans-illuminator (Nippon Genetics) was used to visualise the gel and the desired band was excised using a scalpel.

7.1.2 Digest/PCR product purification

7.1.2.1 Column purification

For column purification, DNA/PCR products were purified using either the QIAquick PCR Purification Kit (#28104, QIAGEN) or the Monarch® PCR & DNA Cleanup Kit (5 µg) (#T1030S, NEB), following the manufacturer's protocol.

7.1.2.2 Gel extraction

DNA/PCR products were extracted from agarose gel using the QIAquick Gel Extraction Kit (#28704, QIAGEN) or Monarch® DNA Gel Extraction Kit (#T1020S, NEB), following the manufacturer's protocol.

7.1.3 Plasmid amplification and purification

7.1.3.1 Chemical transformation

Plasmid DNA was transformed into OneShot™ TOP10 Chemically Competent *E. coli* (#C404003, Invitrogen, ThermoFisher) following the manufacturer's rapid chemical transformation procedure and plated on Luria broth (LB) agar plates containing 100 µg/ml ampicillin. After overnight incubation at 37°C, single colonies were inoculated into liquid LB with 100 µg/ml ampicillin and incubated overnight in a shaking incubator at 37°C, 225 rpm.

7.1.3.2 Glycerol stocks

After overnight incubation, 500 µl of bacterial culture was mixed with 500 µl of 50% glycerol, snap frozen in dry ice and stored at -80°C.

7.1.3.3 Purification

Plasmid DNA was extracted from bacterial cultures using QIAGEN Plasmid *Plus* DNA purification kits (Mini, Midi, Maxi or Mega) following the manufacturer's protocol.

7.1.4 Gibson Assembly

Reactions were set up on ice with a total volume of 20 µl (components specified in the relevant sections) using the Gibson Assembly® MasterMix (#E2611S, NEB). A control reaction was always set up in parallel with water replacing the insert, to give an indication of how successful the cloning had been. Reactions were incubated at 50°C for 1 hour then diluted 1:4 in nuclease-free water and 2 µl of each was chemically transformed into 50 µl of OneShot™ TOP10 Chemically Competent *E. coli* (Section 7.1.3.1). At least 4 colonies from the transformation plate were inoculated for minipreps and plasmid DNA was purified.

7.2 iPSC culture

BOB is a male human iPSC line re-programmed from fibroblasts using Sendaiviral vectors. The cells originally carried a single point mutation in the *A1AT* gene, which was corrected to wildtype using zinc finger nuclease technology[245]. A reciprocal translocation, t(6;8)(p21.1;8q24.1), is present but the karyotype is stable. This line was established at the WSI and it has been used and characterised extensively within the institute. All BOB cell lines (parental and KO lines) were kindly provided by the Gene Editing facility at WSI. These were grown in feeder-free conditions in TeSR™-E8™ medium (#05990, STEMCELL) at 37^{o}C, 5% CO_2 and medium was changed every day. Details provided here are relevant to all iPSC culture discussed in subsequent sections.

7.2.1 Coating culture dishes

Cells were maintained in culture dishes coated in either Synthemax®-II-SC (#CLS3535, Corning) or Vitronectin-XF™ (#07180, STEMCELL). Synthemax®-II-SC powder was resuspended in sterile cell culture water to 1 mg/ml. From this stock, it was further diluted to 25 µg/ml in sterile cell culture water, added to the dish (10 ml/10 cm^2 dish) and incubated at room temperature for 2 hours. Vitronectin-XF™ was diluted to 10 µg/ml in PBS or sterile water, added to the dish (6 ml/10 cm^2 dish) and incubated at room temperature for 1 hour. In both cases, coated dishes were used on the day or stored at 4^{o}C in parafilm for up to 3 days.

7.2.2 Thawing BOB lines

Cryovials were briefly warmed in a 37^{o}C water bath to thaw the cell suspension. Cells were transferred to a Falcon containing a 4-fold excess of TeSR-E8 medium. Centrifugation was carried out at 300 g for 3 minutes and the supernatant was removed. Cells were resuspended in 4 ml of medium plus 10 µM ROCK inhibitor (Y-27632, #72304, STEMCELL). Coating solution was aspirated from a 6 cm^2 dish and the cell suspension was transferred to the dish.

7.2.3 Passaging BOB lines as single cells

All values given are for a 6 cm^2 dish; these were scaled based on surface area as required. For general maintenance, cells were passaged every 6-7 days once dense colonies had formed. Medium was aspirated and cells were washed once with 4 ml of PBS. Cells were incubated

with 2 ml of accutase at 37ºC for 5-10 minutes until cells detached. An equal volume of TeSR-E8 medium was added, cells were pipetted 5-10 times to make a single cell suspension and transferred to a Falcon tube. A small aliquot of cells was mixed 1:1 with Trypan blue. A live cell count was calculated using a Countess™ II Automated Cell Counter (ThermoFisher). Cells were centrifuged at 300 g for 3 minutes and the supernatant was removed. Cells were resuspended in 2 ml of fresh medium. Coating solution was aspirated from the culture dish and 10 ml of medium was added with 10 µM ROCK inhibitor. The desired number of cells were transferred to the culture dish and incubated at 37ºC, 5% CO_2.

7.2.4 Freezing BOB lines

Cells were processed as described in Section 7.2.3. After resuspension, the desired number of cells (generally $2x10^5$ cells/vial) were transferred to a Falcon tube containing KnockOut™ Serum Replacement (KOSR, #10828028, Gibco, ThermoFisher) to a total volume of 500 µl/vial. Another Falcon tube was prepared with 500 µl/vial of KOSR + 20% DMSO. 500 µl of both solutions was added to each vial and inverted several times to mix. Cryovials were stored in a polystyrene rack at -80ºC for 48 hours before transfer to liquid nitrogen.

7.3 Engineering knockout iPSC lines

All KO BOB lines were produced by the Gene Editing facility at WSI. An asymmetrical exon in the gene of interest was substituted with a puromycin cassette and a frameshift indel was introduced into the other allele. A template vector with an EF1a-puromycin cassette was cloned for each gene, with two 1.5 kb homology arms designed to match the sequence around the targeted exon. Two gRNAs targeting each exon were designed (Appendix A.1). The template vector (2 µg), both gRNA vectors (3 µg) and hSpCas9 (4 µg) were transfected into $2x10^6$ cells using the Human Stem Cell Nucleofector® Kit 2 (#VPH-5022, Lonza). Cells were plated in 10 cm^2 dishes and after 72 hours, cells were selected with 3 µg/ml puromycin. Single cells were expanded and genotyped for the presence of a frameshift indel by Sanger sequencing. I repeated genotyping for all lines detailed in this thesis (described in Section 7.4). KO lines were maintained in 1 µg/ml puromycin (#ant-pr-1, InvivoGen).

7.4 Genotyping of knockout lines

7.4.1 DNA extraction

For genotyping, gDNA was extracted from cell pellets using the Gentra Puregene Core Kit A (QIAGEN) following the manufacturer's protocol. DNA was quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer or a Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer.

7.4.2 Genotyping PCR

For each KO gene (in iPSCs and HAP1 cells), primers were designed to cover the edited region (Appendix A.1). PCR was performed on gDNA extracted from all edited lines using Platinum™ *Taq* DNA Polymerase High Fidelity (#11304011, Invitrogen, ThermoFisher) (Table 7.1). PCR products were visualised on an agarose gel (Section 7.1.1) to confirm the correct band was present. These were then column purified (Section 7.1.2.1) and sent for Sanger sequencing (Eurofins) (Appendix A.1).

Table 7.1. Genotyping PCR. Top - reaction composition, bottom - thermocycler conditions.

Reagent	Volume/amount
10X buffer	2.5 µl
$MgSO_4$	1 µl
10 mM dNTP	0.5 µl
10 µM forward primer	0.5 µl
10 µM reverse primer	0.5 µl
Taq polymerase enzyme	0.1 µl
DNA	~500 ng
Nuclease-free water	to 25 µl total

Cycle Number	Denature	Annealing	Extension
1	94˚C, 30 seconds		
2-31 (30 cycles total)	94˚C, 10 seconds	Varied with primer T_m, 30 seconds	68˚C, 1 min/kb

7.5 Protein-level knockout confirmation

7.5.1 Protein lysis

Cells were washed twice with ice-cold PBS then lysis buffer was added (RIPA buffer (#20-188, Merck) diluted in distilled water, plus 1:100 protease/phosphatase inhibitor (#5872, Cell Signaling Technology)); the volume was dependent on cell number. Cells were incubated on ice for 5-10 minutes then scraped and transferred to Eppendorfs. Samples were centrifuged at 14,000 g for 15 minutes at 4^{o}C. Supernatant was transferred to fresh Eppendorfs and stored at -80^{o}C.

7.5.2 Protein quantification

Protein lysates were quantified using the Pierce™ BCA Protein Assay Kit (#23227, Thermo Scientific), following the manufacturer's microplate protocol. Absorbance was measured using a Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer.

7.5.3 Protein separation

SDS-PAGE was performed to separate proteins. NuPAGE™ LDS Sample Buffer (#NP0007, ThermoFisher Scientific) and NuPAGE™ Sample Reducing Agent (#NP0009, ThermoFisher Scientific) were mixed with protein lysate. Samples were heated to 70^{o}C for 10 minutes then placed on ice prior to loading into a pre-cast gel (NuPAGE™ Novex 4-12% Bis-Tris Gel, 1.5 mm x 15 well, #NP0336BOX, ThermoFisher). A protein molecular weight marker was also loaded. The gel was set up in an XCell SureLock™ Mini-Cell and covered with 1X NuPAGE™ MOPS SDS Running Buffer (#NP0001, ThermoFisher) supplemented with NuPAGE™ Antioxidant (500 µl/200 ml, #NP0005, ThermoFisher). Samples were run at 150 V until sufficient separation was achieved.

7.5.4 Western blotting

NuPAGE™ Transfer Buffer (#NP0006, ThermoFisher) was diluted in deionised water and supplemented with NuPAGE™ Antioxidant (1%) and methanol (10%). PVDF membrane was briefly soaked in methanol and placed on top of the gel, assembled with filter paper and blotting pads in an XCell II™ Blot Module. The module was filled with the prepared transfer buffer

and run at 30 V for 1 h. The membrane was then incubated in blocking buffer (5% non-fat milk) dissolved in Tris-Buffered Saline supplemented with 0.1% Tween-20 (TBS-T). Incubation was carried out for 20 minutes - 2 hours at room temperature on a shaker. The membranes were then incubated with primary antibody (diluted in blocking buffer, Table 7.2) overnight at 4^{o}C on a shaker. Five washes were carried out with TBS-T for 5 minutes at room temperature. The membrane was then incubated with secondary antibody (diluted in blocking buffer, Table 7.2) at room temperature for 1 hour on a shaker. Finally, the membrane was washed again 5 times with TBS-T for 5 minutes. To visualise, the membrane was sprayed with ECL reagent (#K-12049-D50, Advansta) and incubated for 1 minute before reading on an ImageQuant™ LAS 4000 using chemiluminescence.

Table 7.2. Antibodies for Western blotting.

Antibody	Supplier	Product #	Dilution (in 5% milk)	Secondary antibody
Primary antibodies				
Arid1b	Abcam	ab57461	1:1000	Goat anti-mouse
Arid2	SantaCruz	sc-166117	1:1000	Goat anti-mouse
Arid1a (PSG3)	SantaCruz	sc-32761	1:1000	Goat anti-mouse
β-Actin (8H10D10)	Cell Signalling Technology	#3700	1:1000	Goat anti-mouse
Secondary antibody				
Goat anti-mouse IgG H&L (HRP)	Abcam	ab97023	1:10,000	-

7.6 Proteomics

Liquid chromatography-mass spectrometry was performed on the parental BOB line and KO derivatives, by Clara Alsinet (previous postdoctoral fellow in the Adams' lab) in collaboration with Jyoti Choudhary and Theodoros Roumeliotis (previously in the Proteomics facility at WSI). Sample preparation and processing were carried out as described in.[324] Data for the parental BOB line and *ARID1A/ARID2*/*PBRM1* KO clones was published in that manuscript as part of a separate project.

7.7 Cloning of a neomycin-resistant gRNA library

7.7.1 Insertion of neomycin resistance gene

Empty Yusa v1.1 library backbone DNA (Addgene #67974) was kindly provided by Fiona Behan (postdoctoral fellow in the Garnett lab, WSI). The backbone was digested with Kpn2I at 37°C for 2 hours (Table 7.3), visualised on an agarose gel to confirm digestion (Section 7.1.1) and then column purified (Section 7.1.2.1). A 901 bp DNA fragment (fragment A, Appendix A.1) (GeneArt Strings, ThermoFisher) was designed, containing a neomycin resistance gene coding region and sequence complementary to the backbone. An additional 120 bp DNA oligonucleotide (fragment B, Appendix A.1) (Ultramer®, Integrated DNA Technologies) was also designed to extend the overlap upstream of the initial Kpn2I site in the PGK promoter. Both fragments were cloned into the digested backbone by Gibson Assembly (Table 7.3) (Section 7.1.4). DNA from four colonies was sent for Sanger sequencing (Eurofins) with six primers which together provided sequence covering the full edited region (Appendix A.1). This backbone is referred to hereafter as 'neoR'.

Table 7.3. Cloning to insert neomycin resistance gene. Top - restriction digest, bottom - Gibson Assembly.

Reagent	Volume/amount
NEB 3.1 buffer	15 µl
Kpn2I (BspEI) (#R0540S, NEB)	10 µl
Yusa v1.1 backbone	10 µg
Nuclease-free water	to 150 µl total

Reagent	Volume/amount
Kpn2I-digested backbone	195.8 ng
Fragment A	111.4 ng
Fragment B	14.84 ng
2X Gibson Assembly MM	10 µl
Nuclease-free water	to 20 µl total

7.7.2 Testing the neoR library backbone

The neoR library backbone was packaged into a lentivirus and titred (Section 7.8). BOB-Cas9 cells were detached and counted. Cells were transduced with the lentivirus at an MOI of 0.3, and 200,000 cells/well were seeded in a 6-well plate with 2 ml total volume. Untransduced cells were also seeded in parallel. Fresh medium was added the following morning. After 48 hours, cells were passaged and a sample was fixed and analysed by flow cytometry to measure BFP expression (Section 7.9). For a clonogenic assay, 10,000 transduced cells were seeded per well in 5 wells of a 6-well plate. One well was seeded with untransduced cells. On the day after seeding, 0, 0.1, 0.3, 0.6 or 1 mg/ml G418 was added to the transduced cells. The same experiment was repeated with 10,000 cells/well in a 12-well plate to allow for further flow cytometry analysis. After 5 days in G418, media was aspirated from the 6-well plate and cells were washed twice with ice-cold PBS. Cells were incubated in 4% paraformaldehyde (PFA) for 20 minutes at room temperature and then washed twice with PBS. Crystal violet (0.5% in methanol) was then added to each well, incubated briefly and then wells were washed with water. Images were taken after plates had dried. After 5 days in G418, cells in the 12-well plate were fixed and BFP expression was measured by flow cytometry (Section 7.9).

7.7.3 Insertion of AjuI restriction sites

The neoR backbone was digested with BbsI at 37ºC for 16 hours (Table 7.4), visualised on an agarose gel to confirm digestion (Section 7.1.1) and the linearised band was gel extracted (Section 7.1.2.2). A 94 base DNA oligonucleotide (fragment C, Appendix A.1) (Ultramer®, Integrated DNA Technologies) was designed to contain the same sequence as the backbone but replacing the BbsI recognition site with AjuI restriction sites. The fragment was inserted into the digested backbone by Gibson Assembly (Table 7.4) (Section 7.1.4). DNA from six colonies was sent for Sanger sequencing (Eurofins) with primers covering the edited region (Appendix A.1).

Table 7.4. Cloning to insert AjuI sites. Top - restriction digest, bottom - Gibson Assembly.

Reagent	Volume/amount
NEB 2.1 buffer	10 µl
BbsI (#R0539S, NEB)	4 µl
neoT2A backbone	10 µg
Nuclease-free water	to 100 µl total

Reagent	Volume/amount
BbsI-digested backbone	435 ng
Fragment C	11.62 ng
2X Gibson Assembly MM	10 µl
Nuclease-free water	to 20 µl total

7.7.4 Insertion of IRES element

PCR was performed to amplify a region containing part of the neomycin resistance gene and an IRES sequence (Table 7.5, primers detailed in Appendix A.1) from an unpublished plasmid kindly provided by Luca Crepaldi (Parts' lab, WSI). 5 µl of the reaction was visualised on an agarose gel (Section 7.1.1) to confirm the correct product size was amplified and then the remainder was column purified (Section 7.1.2.1).

Table 7.5. Amplification of neomycin resistance gene and IRES sequence. Top - reaction composition, bottom - thermocycler conditions.

Reagent	Volume/amount
KAPA HF HS 2X MM (#KK2601, Kapa Biosystems)	12.5 µl
5 µM forward primer	1.5 µl
5 µM reverse primer	1.5 µl
Plasmid DNA	1 ng
Nuclease-free water	to 25 µl total

Cycle Number	Denature	Annealing	Extension
1	95˚C, 3 minutes		
2-31 (30 cycles total)	98˚C, 20 seconds	60˚C, 15 seconds	72˚C, 15 seconds
32			94˚C, 1 minute

An edited clone containing the AjuI sites (Section 7.7.3) was then digested with RsrII and BsrGI at 37°C for 16 hours (Table 7.6). The backbone was visualised on an agarose gel (Section 7.1.1) to confirm digestion and then column purified (Section 7.1.2.1). A 123 bp DNA oligonucleotide (fragment E, Appendix A.1) (Ultramer®, Integrated DNA Technologies) was designed with an overlap between the IRES sequence and the BFP coding region in the backbone. Gibson Assembly was performed to insert the neo-IRES PCR amplicon and the DNA oligonucleotide into the digested backbone (Table 7.6) (Section 7.1.4). DNA from twelve colonies was sent for Sanger sequencing (Eurofins) with primers covering the edited region (Appendix A.1). One successfully edited clone was taken forward, referred to hereafter as 'neoR-IRES'.

Table 7.6. Cloning to insert IRES element. Top - restriction digest, bottom - Gibson Assembly.

Reagent	Volume/amount
10X NEB CutSmart buffer	10 µl
RsrII (#R0501S, NEB)	2 µl
BsrGI-HF (#R3575S, NEB)	2 µl
neoR(AjuI) backbone	5 µg
Nuclease-free water	to 100 µl total

Reagent	Volume/amount
RsrII/BsrGI-digested backbone	106.2 ng
Fragment D	29.5 ng
Fragment E	7.6 ng
2X Gibson Assembly MM	10 µl
Nuclease-free water	to 20 µl total

7.7.5 AjuI digestion of backbone

Various protocols were trialled to optimise digestion of the neoR-IRES backbone with AjuI. Both AjuI (#ER1951, ThermoFisher) and FastDigest AjuI (#FD1954, ThermoFisher) were tested with varying amounts of DNA, DNA:enzyme unit ratios, total reaction volumes and incubation times (from 2-16 hours). An example reaction setup is shown in Table 7.7 (reactions were always incubated at 37°C). Digests were visualised on an agarose gel (Section 7.1.1) then the digested product was gel extracted and purified (Section 7.1.2.2). 10 ng of digested and

undigested plasmid were transformed into OneShotTOP10 cells (Section 7.1.3.1). Colonies were counted after overnight incubation and the % of background was calculated as the number of colonies present with digested plasmid relative to undigested plasmid.

Table 7.7. Restriction digest of neoIRES backbone with AjuI *(or FastDigest AjuI).*

Reagent	Volume/amount
10X Buffer R *(10X FastDigest Buffer)*	5 µl
50X SAM *(20X SAM)*	to 1X
AjuI *(FastDigest AjuI)*	1 µl
neoR-IRES backbone	1 µg
Nuclease-free water	to 20 µl total

To confirm that AjuI was cutting at both restriction sites, PCR was carried out to amplify a 194 bp covering the region (Table 7.8) (Appendix A.1). The product was column purified (Section 7.1.2.1) and digested with FastDigest AjuI (Table 7.7). Digested and undigested PCR products were visualised an agarose gel (Section 7.1.1).

Table 7.8. Amplification of AjuI sites. Top - reaction composition, bottom - thermocycler conditions.

Reagent	Volume/amount
Q5 HS HF 2X MM	12.5 µl
10 µM forward primer	1.25 µl
10 µM reverse primer	1.25 µl
neoIRES backbone	1 µg
Nuclease-free water	to 25 µl total

Cycle Number	Denature	Annealing	Extension
1	98˚C, 30 seconds		
2-31 (30 cycles total)	98˚C, 10 seconds	67˚C, 30 seconds	72˚C, 30 seconds
17			72˚C, 2 minutes

7.7.6 Testing the neoR-IRES library backbone

The neoR-IRES library backbone was tested as described in Section 7.7.2. The neoR library backbone was also tested again in parallel for comparison, using the 6-well clonogenic assay.

7.7.7 MluI/AgeI digestion of backbone

The neoR-IRES library backbone was digested with MluI and AgeI to prepare for transfer of gRNAs (Table 7.9). Digestion was carried out at 37ºC for 8 hours, then the reaction was visualised on an agarose gel (Section 7.1.1) and the digested product was gel extracted and purified (Section 7.1.2.2). To confirm efficient digestion, 10 ng of digested and undigested plasmid were transformed into OneShotTOP10 cells (Section 7.1.3.1). Colonies were counted after overnight incubation and the % of background was calculated as the number of colonies present with digested plasmid relative to undigested plasmid.

Table 7.9. Restriction digest of neoIRES backbone with MluI/AgeI.

Reagent	Volume/amount
10X NEB CutSmart Buffer	30 µl
AgeI-HF (#R3552S, NEB)	5 µl
MluI-HF (#R3198S, NEB)	7.5 µl
neoIRES backbone	10 µg
Nuclease-free water	to 300 µl total

7.7.8 PCR of existing genome-wide gRNA library

DNA for the Yusa v1.1 library was kindly provided by Fiona Behan (Garnett lab, WSI). To amplify gRNAs from the library, PCR was performed using primers that covered the gRNA and extended across the MluI/AgeI restriction sites (Table 7.10, primers detailed in Appendix A.1). An initial optimisation experiment was carried out to identify the best annealing temperature and primer set. Two primer sets were tested as described in Table 7.10, with six annealing temperatures between 66-71ºC. 5 µl of each PCR product was visualised on an agarose gel (Section 7.1.1) and the condition with the strongest band was chosen (67ºC annealing temperature with primer set 2). Using these optimised conditions, 11 PCR reactions were performed with 100 ng of library DNA input per reaction. All reactions were pooled and visualised on an agarose gel (Section 7.1.1). The 599 bp band was then gel extracted and purified (Section 7.1.2.2).

Table 7.10. Amplification of gRNAs from Yusa v1.1 library. Top - reaction composition, bottom - thermocycler conditions.

Reagent	Volume/amount
Q5 HS HF 2X MM (#M0494S, NEB)	12.5 µl
10 µM forward primer	1.25 µl
10 µM reverse primer	1.25 µl
Yusa v1.1 library DNA	100 ng
Nuclease-free water	to 25 µl total

Cycle Number	Denature	Annealing	Extension
1	98˚C, 30 seconds		
2-16 (15 cycles total)	98˚C, 10 seconds	67˚C, 30 seconds	72˚C, 30 seconds
17			72˚C, 2 minutes

7.7.9 Gibson Assembly to clone gRNA library

Twelve Gibson Assembly reactions were performed to insert the gRNA fragment into the MluI/AgeI digested backbone (Table 7.11, Section 7.1.4). All reactions were pooled and column purified using the Monarch kit (Section 7.1.2.1), with a 12 µl final elution volume.

Table 7.11. Gibson Assembly reaction to clone gRNA library.

Reagent	Volume/amount
Digested backbone	439.2 ng
gRNA PCR product	88.84 ng
2X Gibson Assembly MM	10 µl
Nuclease-free water	to 20 µl total

7.7.10 Bacterial amplification of gRNA library

Endura ElectroCompetent cells (#60242-1, Lucigen) were thawed on ice and 25 µl of cells was added to 2 µl of purified Gibson Assembly product in a chilled Eppendorf. This was transferred to a chilled 1.0 mm electroporation cuvette, ensuring even distribution and no bubbles. Electroporation was performed with the following parameters: 10 µF, 600 Ohms, 1800 Volts. Immediately after the pulse, 975 µl of recovery medium was added and cells were transferred

to a 15 ml BD Falcon polystyrene tube with snap-cap. Cells were incubated at 37ºC, 225 rpm for 1 hour. This was repeated 9 times and all cultures were pooled after incubation.

For colony counting to estimate transformation efficiency, dilutions of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were prepared and 50/100/150 µl of each were plated on LB agar plates containing 100 µg/ml ampicillin. These were incubated for 16 hours at 37ºC. The rest of the culture was divided between 6 flasks, each containing 500 ml of liquid LB plus 100 µg/ml ampicillin. These were incubated for 16 hours at 37ºC, 225 rpm.

Colonies were counted on each dilution plate and an average value was calculated and extrapolated to the total culture volume, giving an estimate of the library coverage achieved. Bacterial stabs for 94 colonies were made in a 96-well plate containing LB agar with 100 µg/ml ampicillin. The plate was incubated for 16 hours at 37ºC and sent for plasmid purification and Sanger sequencing (Eurofins) with primers covering the gRNA region (Appendix A.1).
The liquid cultures were pooled and split into 12x 250 ml centrifuge bottles. Centrifugation was performed at 6000 g for 15 minutes at 4ºC. The pellets were resuspended, pooled and transferred to 15 ml Falcons then centrifugation was repeated. Supernatant was removed and plasmid purification was performed using Megaprep and Maxiprep kits (Section 7.1.3.3). All DNA was pooled and quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer.

Details of PCR amplification, purification and sequencing of the gRNAs in the resulting neoR-IRES library are provided in Section 7.12.

7.8 Lentivirus production and titration

7.8.1 Lentivirus production

All values given are for lentivirus production in a T150 flask (Table 7.12); these were scaled according to surface area for different culture dishes. HEK293T cells were cultured in IMDM (#12-722F, Lonza) supplemented with 10% Fetal Bovine Serum (FBS) (#A3160401, Gibco, ThermoFisher) and incubated at 37°C, 5% CO_2. Cells were seeded at a density of $18x10^6$ cells/T150 24 hours prior to transfection. Immediately before transfection, medium was aspirated and replaced with 12.5 ml Opti-MEM™ (#31985062, Gibco, ThermoFisher). Transfer vector and packaging plasmids pMD2.G (Addgene #12259) and psPAX2 (Addgene #12260) were diluted in Opti-MEM™. PLUS™ reagent (#15338100, ThermoFisher) was then added and the mixture was inverted 10-15x and incubated for 5 minutes at room temperature. Lipofectamine™ LTX (#15338100, ThermoFisher) was added, the solution was inverted 10-15x and incubated for 30 minutes at room temperature. The lipofectamine/DNA complex was then carefully added to the cells. After 16 hours, virus medium was aspirated and replaced with 24 ml complete IMDM. Approximately 30 hours later, virus supernatant was collected and filtered with a 0.45 µm low protein-binding filter (#SLHP033RS, Merck). Aliquots were stored at -80°C and were frozen for at least 2 hours before being used in experiments.

Table 7.12. Composition of lentiviral transfection complex.

Reagent	Volume/amount
Opti-MEM™	7.5 ml
Transfer vector	7.5 µg
psPAX2	18.5 µg
pMD2.G	4 µg
PLUS™ reagent	30 µl
Lipofectamine™ LTX	90 µl

7.8.2 Lentivirus titration

All lentiviruses described in this thesis were titrated by measuring fluorescent marker expression using flow cytometry (Section 7.9). Cells were detached and diluted to the concentration that would be required for the assay in which the lentiviruses would be used. For HAP1 cells, polybrene was added to a final concentration of 8 µg/ml; for iPSCs no polybrene

was used. Cell suspension was then added to a 6-well plate and varying volumes of virus were added to each well. As a control, one well was cultured without virus. After 48 hours, cells were detached and prepared for flow cytometry (Section 7.9). Untransduced cells were used to set a gate for fluorescent marker expression. The % of positive cells was used as an indicator of the MOI at each dose, and a line of best fit was plotted to allow calculation of the virus volume required for any given MOI. The amount of virus, cell number and total volume were then scaled based on the culture surface area used in further experimental transductions.

7.9 Analysis of cells by flow cytometry

7.9.1 Cell fixation

iPSCs or HAP1 cells were detached, transferred to an Eppendorf and centrifuged at 300 g for 3 minutes. Supernatant was removed and cells were washed with 500 µl PBS to remove residual medium. Centrifugation was repeated (300 g, 3 minutes), PBS was removed and cells were resuspended in 100 µl of 4% PFA (#F8775, Sigma). Fixation was carried out at room temperature for 15 - 20 minutes, then 500 µl PBS was added. Cells were centrifuged (300 g, 3 minutes) and the supernatant was discarded. Cells were resuspended in 500 µl PBS, centrifuged (300 g, 3 minutes) and supernatant was removed. Finally, cells were resuspended in PBS (volume dependent on pellet size, generally ~ 500 µl) and transferred to a Falcon® 5 ml Round Bottom Polystyrene Tube (#352054, Corning).

7.9.2 Flow cytometry analysis

Cells were analysed using a BD LSRFortessa™ (or occasionally BD LSRII) with BD FACSDiva™ software. Expression of BFP (405 (450/50)) and GFP (488 (530/30)) were detected on the Fortessa or LSRII, and mCherry (561 (610/20)) was detected only on the Fortessa. Results were then further analysed and plotted using FlowJo.

7.10 Engineering stable Cas9 lines

7.10.1 Cas9 transduction

A plasmid expressing Cas9 and blasticidin resistance (Addgene #68343) was packaged into a lentivirus (Section 7.8.1). Cells were detached and transduced in suspension. For iPSC lines, $7.5x10^5$ cells were transduced with 450 µl of lentivirus and seeded in a 6 cm^2 dish. For HAP1 cells, $5x10^5$ cells were transduced with 100 µl of lentivirus plus 8 µg/ml polybrene and seeded in a T25 flask. In both cases, control cells containing no lentivirus were seeded in parallel. The following morning, fresh complete medium was added. At 48 hours post-transduction, 10 µg/ml blasticidin was added (#antbl-1, InvivoGen). Complete death in the control flask confirmed successful blasticidin selection. All iPSC and HAP1 Cas9-expressing cell lines were continuously cultured in blasticidin, except during screens, to ensure high Cas9 activity was maintained. After ~2 weeks of selection, Cas9 activity was assessed (Section 7.10.2) to confirm that lines were functional before freezing stocks and using in further assays.

7.10.2 Quantification of Cas9 activity

Two vectors were used to assess Cas9 activity; one expressing BFP and GFP (control, Addgene #67979) and one expressing BFP, GFP and a gRNA targeting GFP (reporter, Addgene #67980). Both vectors were packaged into lentiviruses (Section 7.8.1). Cells were detached and transduced in suspension; one condition with no lentivirus, one with the control and one with the reporter. For iPSC lines, $2x10^5$ cells/well were seeded in a 12-well plate with 50 µl of lentivirus. For HAP1 lines, $2x10^5$ cells/well were seeded in a 6-well plate with 100 µl of lentivirus and 8 µg/ml polybrene. Fresh complete medium was added the following morning and after 48 hours cells were fixed and analysed by flow cytometry (Section 7.9). BFP and GFP expression were measured, using untransduced cells and cells transduced with the control vector to set gates for both markers.

7.11 NeoR-IRES library screen in iPSCs

7.11.1 Library transduction

The neoR-IRES gRNA library was packaged into a lentivirus and titred in all stable Cas9 iPSC lines (parental and KOs) (Section 7.8). For each screened line, cells were split into three separate populations at the passage prior to transduction. Each population was then detached and counted as single cells, and three separate transduction replicates were set up. For each replicate, a total of $54x10^6$ cells were transduced with the library lentivirus at an MOI of 0.3 in 594 ml total ($50x10^6$ cells were seeded but extra cells were transduced to allow for volume lost in pipetting). Cells were then seeded at $2x10^6$ cells/dish in a 15 cm dish (25 dishes total, for screens I performed) or $3.4x10^6$ cells in a T225 (17 flasks total, for screens CGaP performed). A small sample (3 ml of transduced cell suspension) was seeded in a 6 cm^2 dish for each replicate, and untransduced cells were also seeded in parallel at the same density. After 48 hours, 1 mg/ml G418 was added to all transduced cells. The small samples of transduced and untransduced cells were fixed and BFP expression was analysed by flow cytometry to measure transduction rate (Section 7.9). Untransduced cells were re-seeded and maintained in parallel with the screens.

7.11.2 Passage of cells during screen

For the screens that I performed, cells were passaged as clumps on day 8 post-transduction. Medium was aspirated and cells were washed with PBS. Gentle Dissociation Reagent (#07174, STEMCELL) was added (10 ml/15 cm dish) and cells were incubated at 37ºC for 10 minutes. An equal volume of TeSR-E8 was then added and the suspension was mixed no more than 5 times with a stripette. All cells were combined in a single bottle for each replicate and mixed gently by inversion.

To give a more accurate estimate of cell count, 3 ml of cell suspension was transferred to 1.5 ml Eppendorfs (1 ml/Eppendorf) and mixed further to dissociate clumps to single cells. Cells were centrifuged for 3 minutes at 300 g, media was aspirated and 500 µl of accutase was added. Cells were incubated in the Eppendorfs for 5-10 minutes at 37ºC. An equal volume of TeSR-E8 was added and 10 µl from each Eppendorf was mixed with Trypan blue and counted using a Countess™ II Automated Cell Counter. Based on the average cell count, cells were

diluted in TeSR-E8 and $50x10^6$ cells total were maintained per replicate. Cells were seeded at the same density of $2x10^6$ cells/152 cm^2, but in 5x 5-layer flasks (#11597421, Corning).

For screens that CGaP performed, the same protocol was followed but cells were passaged as clumps on day 6 post-transduction, maintaining $100x10^6$ cells total per replicate in 10x T225 flasks. Cells were then passaged as clumps again on day 8/9 post-transduction, maintaining $50x10^6$ cells total per replicate in 5x 5-layer flasks (#11597421, Corning).

At the point of passage, a small sample of cells from each replicate was fixed and BFP expression was analysed by flow cytometry (Section 7.9). Untransduced cells that had been maintained in parallel were also fixed and analysed as a control.

7.11.3 Harvesting of cells at screen endpoint

Cells were maintained in 1 mg/ml G418 until day 13 post-transduction. Cells were then detached as single cells using accutase, counted and $60x10^6$ cells were transferred to a 50 ml Falcon. A small sample of cells from each replicate was also fixed and BFP expression was analysed by flow cytometry (Section 7.9). Untransduced cells that had been maintained in parallel were also fixed and analysed as a control. Cells were centrifuged at 300 g for 3 minutes and the supernatant was aspirated. Pellets were resuspended in PBS, transferred to a 15 ml Falcon and centrifuged again. PBS was aspirated and the pellets were stored at -80^{o}C until DNA extraction.

7.12 Sequencing of neoR-IRES library gRNAs

7.12.1 DNA extraction

For each screen replicate, gDNA was extracted from pellets of $60x10^6$ cells using a Blood & Cell Culture DNA Maxi Kit, with each pellet split over two columns (#13362, QIAGEN).

7.12.2 First round PCR & purification

PCR was performed to amplify the gRNA region of the gDNA in each screen sample and the plasmid DNA from the neoR-IRES library (Table 7.13, primers detailed in Appendix A.1). A total of 72 PCR reactions with 2 µg gDNA/reaction were performed for every screen sample to ensure high representation of the library. For sequencing of the library from plasmid DNA, the same PCR was performed but with 10 reactions and 15 ng plasmid DNA/reaction.

Table 7.13. First round gRNA PCR. Top - reaction composition, bottom - thermocycler conditions.

Reagent	Volume/amount per rxn
Genomic DNA	2 µg
Q5 HS HF 2X MM	25 µl
Primer mix (10 µM of both forward and reverse)	1 µl
Nuclease-free water	to 50 µl total

Cycle Number	Denature	Annealing	Extension
1	98˚C, 30 seconds		
2-29 (28 cycles in total)	98˚C, 10 seconds	61˚C, 15 seconds	72˚C, 20 seconds
30			72˚C, 2 minutes

To confirm successful amplification, 5 µl of each reaction was visualised on an agarose gel (Section 7.1.1). For each sample, 5 µl of all PCR reactions was pooled and purified using a QIAquick PCR Purification Kit (#28104, QIAGEN), following the manufacturer's protocol. The PCR products were then quantified using a High Sensitivity Qubit and diluted to 40 pg/µl.

7.12.3 Second round PCR & purification

A second PCR was performed to add sequencing tags to the initial product (Table 7.14, primers detailed in Appendix A.1).

Table 7.14. Second round gRNA PCR. Top - reaction composition, bottom - thermocycler conditions.

Reagent	Volume
1st PCR Product (40 pg/µl dilution)	5 µl (200 pg)
Primer mix (5 µM of both forward and reverse)	2 µl
Nuclease-free water	18 µl
KAPA HF HS 2X MM (#KK2601, Kapa Biosystems)	25 µl

Cycle Number	Denature	Annealing	Extension
1	98˚C, 30 seconds		
2-9 (8 cycles in total)	98˚C, 10 seconds	66˚C, 15 seconds	72˚C, 20 seconds
10			72˚C, 5 minutes

For each sample, 1 µl of PCR product was run on a Bioanalyser to confirm that the correct band (~300 bp) was present at ~ 2 ng/µl. AMPure SPRI beads were added to the PCR products at a ratio of 0.8:1, mixed by pipetting and incubated at room temperature for 5 minutes. The Eppendorfs were placed on a magnetic rack for 5 minutes to allow the beads to be pulled out of the solution, and the supernatant was discarded. Samples were then washed twice with 200 µl of 80% ethanol for 30 seconds. Ethanol was discarded, the tube was centrifuged briefly to remove residual ethanol and then left to air dry for 5 minutes. The Eppendorfs were removed from the magnet and 35 µl of nuclease-free water was added; samples were left for 3 minutes to allow the DNA to elute. Tubes were placed back on the magnet for 3 minutes and the DNA solution was transferred to a clean Eppendorf. The Bioanalyser was used to confirm that the clean-up was successful and samples were stored at -20ºC prior to sequencing.

Single-end sequencing was performed on an Illumina HiSeq 2500 (primer detailed in Appendix A.1). Generally, six screen samples (replicates from two screens) were multiplexed and run together in two lanes. The neoR-IRES library was sequenced alone in two lanes.

7.12.4 Sequencing data analysis

After sequencing, reads were aligned to the gRNA sequences by the internal CASM pipeline. I then performed all analysis of gRNA sequencing data, with guidance and scripts kindly provided by Vivek Iyer (previous senior bioinformatician, Adams' lab) and Francesco Iorio (group leader, WSI). Details of the data analysis are provided in the relevant results chapters.

7.13 Assays to test library toxicity and expression issues

7.13.1 Comparison of BFP expression in BOB and BOB-Cas9 cells

BOB and BOB-Cas9 cells were detached as single cells, counted and transduced in suspension with the neoR-IRES library lentivirus. Both lines were seeded at $1.8x10^5$ cells/well in a 6-well plate with 0-90 µl of lentivirus and 2 ml total volume. After 72 h, cells were fixed and BFP expression was analysed by flow cytometry (Section 7.9).

7.13.2 Assay to measure BFP expression in single colonies

After completion of genome-wide screening with the neoR-IRES library, a small fraction of BOB-Cas9 cells were maintained in culture. Cells were seeded at a low density and after several days in culture, medium was aspirated and PBS was added. Under the microscope, 18 single colonies were scraped from the dish and transferred to a 24-well plate. Additionally, 2 colonies were picked from a population of untransduced BOB-Cas9 cells cultured for the same period of time These were cultured for 2 weeks and at the point of passage, a sample of cells were fixed and analysed by flow cytometry to measure BFP expression (Section 7.9).

7.13.3 Assay to test cross-resistance to G418

A BOB-Cas9 clonal line with ~100% BFP expression was mixed with an untransduced BOB-Cas9 clonal line (from the assay described in Section 7.13.2). Cells were detached as single cells, counted and combined in various ratios: 0:100, 30:70, 50:50, 75:25, 100:0 (BFP positive:untransduced). From these mixtures, 24,000 cells total/well were seeded in 12-well plates +/- 1 mg/ml G418. The remaining cells for each condition were fixed and analysed by flow cytometry to measure BFP expression (Section 7.9). After 5 days in culture, this analysis was repeated.

7.14 Karyotyping of iPSCs

iPSCs were harvested for karyotyping at 50-70% confluency. First, medium was aspirated and fresh TeSR-E8 medium with 10 μM ROCK inhibitor was added to the cells. Cells were then transferred to Sandra Louzada Gomes Pereira (FISH facility at WSI) to perform the rest of the assay. Nocodazole was added to a final concentration of 25-100 ng/ml and incubated for 2-3 hours at 37°C, 5% CO_2. Medium was transferred to a Falcon and cells were washed with PBS pre-warmed to 37°C. 1 ml of 37°C accutase was added and cells were incubated at 37°C, 5% CO_2 for 3-5 minutes. Cells were resuspended using the medium removed previously and centrifuged at 300 g for 5 minutes. Supernatant was aspirated and cells were resuspended gently in 8-10 ml of hypotonic buffer (0.4% KCl in 10 mM HEPES, pH 7.4). Cells were incubated for 10-20 minutes at 37°C, 5% CO_2. 1 ml of fixative (4:1 methanol:glacial acetic acid) was added and the tube was gently inverted. Cells were centrifuged at 300 g for 5 minutes, supernatant was removed and cells were resuspended. 1 ml of fixative was slowly added, mixed by pipetting and hypotonic buffer was added to a total volume of 10 ml. Cells were centrifuged at 300 g for 5 minutes and supernatant was removed. Cells were resuspended in 1 ml of fixative and 10-12 μl was dropped on to a clean microscope slide. Preparation of human 24-colour M-FISH probes and slide treatments were performed as described in [325,326]. Ten metaphases were analysed per sample.

7.15 Staining for pluripotency markers

This assay was performed with the assistance of Mary Goodwin (CGaP, WSI).

7.15.1 Seeding and fixation

BOB-Cas9 cells (untransduced and cells that had been screened with the neoR-IRES library) were detached as single cells, counted and seeded at 10,000 cells/well in a CELLSTAR® black 96-well plate (#655090, Greiner Bio-One). Cells were fixed after 72 hours: medium was aspirated and 50 μl of 4% PFA (#F8775, Sigma) was added to each well. Fixation was carried out at 4°C for 20 minutes. PFA was aspirated and cells were washed twice with 100 μl/well of PBS for 5 minutes at room temperature. Prior to staining, 100 μl/well of PBS was added, the plate was sealed in parafilm and stored at 4°C.

7.15.2 Staining

PBS was aspirated and 100 µl/well of 1% blocking solution (1g BSA/100 ml of 0.1% Triton X-100 in PBS) was added. Blocking solution was aspirated and 50 µl/well of diluted primary antibody (Table 7.15) was added (four wells/antibody for each cell line). As a control, 50 µl/well of 1% blocking solution was added (two wells/antibody for each cell line). Plates were sealed with parafilm and incubated overnight at 4ºC. DAPI solution (10 mg/ml) was added to each secondary antibody dilution to a final dilution of 1:1000, and these were kept on ice and protected from light. Primary antibody was aspirated and cells were washed three times with 100 µl/well PBS for 5 minutes. 50 µl/well of diluted secondary antibody plus DAPI was added, the plate was wrapped in foil and incubated at room temperature for 1 hour. Secondary antibody was aspirated and cells were washed 3 times with 100 µl/well PBS for 5 minutes. After the final wash, 100 µl/well of PBS was added and plates were sealed with a foil cover and stored at 4ºC prior to analysis.

Cells were analysed using an ArrayScan VTI HCS Reader. For each well, 10,000-12,000 DAPI positive cells were analysed for the presence of OCT4, NANOG or SOX2. The mean % of positive cells was calculated across the 4 technical replicates for each condition and the % of positive cells in the relevant control wells was subtracted to remove any background.

Table 7.15. Antibodies for pluripotency test.

Antibody	Supplier	Product #	Dilution (in 1% blocking solution)	Secondary antibody
Primary antibodies				
Oct4	Santa Cruz Biotech	SC-5279	1:100	Donkey anti-mouse
Sox2	R&D Systems	AF2018	1:100	Donkey anti-goat
Nanog	R&D Systems	AF1997	1:100	Donkey anti-goat
Secondary antibodies				
Donkey anti-mouse (AF647)	Invitrogen	A31571	1:1000	-
Donkey anti-goat (AF488)	Invitrogen	A11055	1:1000	-

7.16 Validation assays

7.16.1 HAP1 cell culture

HAP1 cells (parental and knockout derivatives, Table 7.16) were purchased from Horizon Discovery (Cambridge, UK). All lines were cultured in IMDM (#12440053, Gibco, ThermoFisher) supplemented with 10% FBS at 37°C, 5% CO_2. Knockout lines were genotyped to confirm their mutational status as described in Section 7.4.

Table 7.16. Details of HAP1 cell lines.

Cell line	Mutation	Product code
HAP1 parental control	N/A	C631
HAP1 ARID1A knockout	13 bp deletion in exon 2	HZGHC000618c010
HAP1 ARID1B knockout	13 bp deletion in exon 2	HZGHC000582c007
HAP1 ARID2 knockout	7 bp deletion in exon 3	HZGHC000907c009
HAP1 PBRM1 knockout	8 bp deletion in exon 3	HZGHC001135c010

7.16.1.1 Thawing HAP1 cells

Cryovials were heated in a 37°C water bath to thaw the cell suspension. A 4-fold volume of medium was added and centrifuged at 300 g for 3 minutes. The supernatant was aspirated, cells were resuspended and transferred to a flask containing fresh medium.

7.16.1.2 Passaging HAP1 cells

(All volumes given are for a T75 flask.) Medium was aspirated and cells were washed with 10 ml PBS. To detach cells, 2 ml of trypsin-EDTA (#25200056, Gibco, ThermoFisher) was added and incubated at 37°C for 3-5 minutes. An equal volume of medium was added to neutralise the trypsin and cells were transferred to a new flask, diluted to 1:15 for general maintenance. If an exact density was required, 10 µl of cell suspension was mixed 1:1 with Trypan Blue and live cell count was then calculated using a Countess™ II Automated Cell Counter.

7.16.1.3 Freezing HAP1 cells

Cells were detached as described above, and centrifuged at 300 g for 3 minutes. The supernatant was aspirated and cells were re-suspended in complete medium supplemented with

20% FBS and 10% DMSO. Cells were transferred to a cryovial and stored in a polystyrene rack at -80°C for 48 hours before being transferred to liquid nitrogen.

7.16.2 Cloning single gRNAs for validation

For validation of the *ARID1A/ARID1B* synthetic lethal interaction, gRNAs were selected from the neoR-IRES genome-wide library. For each gRNA, a pair of single-stranded DNA oligonucleotides containing the gRNA sequence (sense and anti-sense) were ordered, with overlaps to allow for cloning (Appendix A.1). Each oligonucleotide pair was phosphorylated and annealed (Table 7.17) in a thermocycler at 37°C for 30 minutes followed by 95°C for 5 minutes, then left to cool to room temperature. Annealed oligonucleotides were diluted to 7.1 fmol/µl in EB buffer (Qiagen).

Table 7.17. Cloning single gRNA plasmids. Top - annealing gRNA oligonucleotides, bottom left - restriction digest with BbsI, bottom right - ligation of gRNAs into backbone.

Reagent	Volume
100 µM top strand oligo	1 µl
100 µM bottom strand oligo	1 µl
10X T4 ligase buffer	1 µl
T4 PNK enzyme (#M0201, NEB)	0.5 µl
Nuclease-free water	6.5 µl

Reagent	Volume/amount
Plasmid	5 µg
10X CutSmart Buffer	5 µl
BbsI (#R0539S, NEB)	1 µl
Nuclease-free water	to 50 µl total

Reagent	Volume
20 ng/µl digested backbone	1 µl
7.1 fmol/µl annealed oligo	2 µl
10X ligase buffer	1 µl
T4 ligase enzyme (#M0202S, NEB)	1 µl
Nuclease-free water	5 µl

gRNA backbones containing either mCherry (Addgene #67977) or BFP (Addgene #67974) were digested with BbsI for 3 hours at 37°C then heat inactivated at 65°C for 20 minutes (Table 7.17). Digestion was confirmed on an agarose gel (Section 7.1.1) and both vectors were column purified (Section 7.1.2.1) then diluted to 20 ng/µl in nuclease-free water (QIAGEN).

Ligations were performed to insert each gRNA into either the mCherry or BFP backbone; these were incubated at 16ºC for 2 hours (Table 7.17). For each ligation, 2.5 µl was chemically transformed into 25 µl of OneShot TOP10 cells (Section 7.1.3.1). Three colonies per ligation were then inoculated in liquid LB broth + 100 µg/ml ampicillin and plasmid DNA was purified (Section 7.1.3.3). Successful cloning was confirmed by Sanger sequencing (Eurofins) of the gRNA region (Appendix A.1). One clone for each gRNA was packaged into a lentivirus and titred in BOB-Cas9 cells (Section 7.8). An mCherry backbone containing a gRNA targeting *ACCSL* and a BFP backbone containing a gRNA targeting *AIP1L* (the same backbones used for *ARID1A/ARID1B* gRNA cloning) were kindly provided by Nicola Thompson (PhD student in the Adams' lab).

7.16.3 Competitive growth assays for *ARID1A/ARID1B* SLI validation

7.16.3.1 Double gRNA assay

Cells were detached as single cells, counted and transduced in suspension. BOB-Cas9 cells were seeded at a density of 180,000 cells/well in a 6-well plate with 2 ml total volume. Each well was transduced with two lentiviruses, both containing a single gRNA plasmid, aiming for an MOI of ~0.6 for each. The following combinations of gRNA plasmids were transduced: *ARID1B*/*ACCSL*, *ARID1A/AIPL1, ACCSL/AIPL1.* A control well with no lentivirus was also included. Fresh complete medium was added the following morning. After 48 hours, cells were passaged to a new 6-well plate and a sample from each condition was fixed for flow cytometry analysis (Section 7.9). BFP and mCherry expression were measured to establish a baseline level; the % of cells in each population (two single positives and a double positive) was normalised against the untransduced population to calculate relative abundance. Cells were maintained for 2 weeks post-transduction, then were fixed for flow cytometry analysis (Section 7.9). BFP and mCherry expression were measured again and the % of cells in each population was normalised against the untransduced population to calculate relative abundance. The $\log_2$(fold-change) in relative abundance between day 14 and day 2 was calculated, as a measure of change in expression. The expected growth phenotype of the double positive population was calculated as the sum of the changes in both single positive populations.

7.16.3.2 Single gRNA assay

BOB-Cas9, ARID1A_C09-Cas9 and ARID1B_C03-Cas9 lines were seeded and assayed in the same way as described in Section 7.16.3.1, but each line was transduced with only a single gRNA lentivirus per well. BOB-Cas9 cells were transduced with *AIPL1, ARID1A* and *ARID1B* gRNA plasmids. ARID1A_C09-Cas9 cells were transduced with *AIPL1* and *ARID1B* gRNA plasmids. ARID1B_C03-Cas9 cells were transduced with *AIPL1* and *ARID1A* gRNA plasmids. The % of BFP positive cells on day 14 was calculated as a $\log_2$(fold-change) relative to day 2.

7.16.4 Sanger arrayed library gRNAs

An internal facility at WSI hold glycerol stocks for all gRNAs in the Sanger Human Whole Genome CRISPR arrayed library. I provided a 96-well block containing liquid LB broth + 100 µg/ml ampicillin and the facility inoculated each well with a single gRNA plasmid (Appendix A.1). I cultured these overnight in a shaking incubator at 37ºC, 225 rpm, prepared glycerol stocks and purified plasmid DNA (Section 7.1.3). Each plasmid was sent for Sanger sequencing (Eurofins) with a primer complementary to the U6 promoter (Appendix A.1) to confirm the correct gRNA was present. These were then packaged into lentiviruses (Section 7.8.1).

7.16.5 Competitive growth assays for validation of screen hits

Cells were detached, counted and transduced in suspension (as described in Sections 7.2.3 and 7.15.1.2). HAP1-Cas9 cells (parental and KOs) were seeded at a density of 90,000 cells/well in a 12-well plate with 1 ml total volume. BOB-Cas9, ARID1A_C09-Cas9, ARID1B_C03-Cas9, ARID2_C11-Cas9 and PBRM1_F09-Cas9 cells were seeded at 100,000 cells/well in a 12-well plate or 200,000 cells/well in a 6-well plate with 1 ml or 2ml total volume, respectively. Polybrene was added to a final concentration of 8 µg/ml (for HAP1-Cas9 lines only) and each well was transduced with a lentivirus containing a single gRNA plasmid, aiming for an MOI of 0.5-0.8. A control well with no lentivirus was always included. Fresh complete medium was added the following morning. After 48 hours, cells were passaged to a new plate and a sample of each condition was fixed for flow cytometry analysis (Section 7.9). BFP expression was measured to establish a baseline level. Cells were maintained for 2 weeks post-transduction, then cells were fixed for flow cytometry analysis (Section 7.9) (iPSCs did not survive past 1 week). Expression of BFP was measured again on day 14 for HAP1-Cas9 cells and a $\log_2$(fold-change) was calculated relative to day 2, to assess change over time.

7.17 Methods for *in vivo* CRISPRa project

7.17.1 Cell culture

B16-F0-dCas9 cells were cultured in DMEM-high glucose medium (#11965092, Gibco, ThermoFisher), supplemented with 10% FBS, 1% penicillin/streptomycin/glutamine and 15 µg/ml blasticidin. Cells were incubated at 37°C, 5% CO_2. For passaging, medium was aspirated and the cells were washed once with 10 ml PBS (values given for a T150 flask). Cells were then incubated in 5 ml of trypsin-EDTA at 37°C for 5 minutes until they detached. An equal volume of medium was added, cells were re-suspended and seeded in a new flask with fresh medium, generally at a dilution of 1:15-1:30. Cells were counted by mixing 1:1 with trypan blue and analysing live cell number using a Countess™ II Automated Cell Counter (ThermoFisher).

7.17.2 Preparation of the CRISPRa library

The mCRISPRa-v2 m6 subpooled library was obtained from Addgene (#84003), transformed into ElectroMAX™ DH5α-E™ Competent Cells (#11319019, Invitrogen, ThermoFisher) and re-amplified in liquid culture following the Weismann lab protocol (available on the Addgene page). DNA was extracted using a QIAGEN EndoFree Plasmid Mega Kit (#12381, QIAGEN).

7.17.3 Titration of CRISPRa lentiviruses

Lentiviruses were prepared as described in Section 7.8.1. To measure the titre of the lentiviruses, cells were detached and diluted to $9x10^4$ cells/ml. Polybrene was added to a final concentration of 8 µg/ml. 2 ml of cell suspension was then added to a 6-well plate and different volumes of virus were added to each well. As a control, one well was cultured without virus. After 48-72 hours, cells were detached and prepared for flow cytometry (Section 7.9) Untransduced cells were used to set a gate for BFP expression. The % of BFP positive cells was used as an indicator of the MOI at each dose, and a line of best fit was plotted to allow calculation of the volume required for any given MOI. The amount of virus, cell number and total volume were scaled based on the culture surface area used in further transductions.

7.17.4 CRISPRa screen

7.17.4.1 *In vitro* phase

A total of $12x10^6$ cells were transduced with the m6 library lentivirus at an MOI of 0.3 in 180 ml total volume with 8 μg/ml polybrene. The cell suspension was split into 6x T150 flasks. A culture of untransduced cells was maintained in parallel as a control. Medium was changed the following day. Cells were passaged after 48 hours and $16x10^6$ cells were re-seeded in 5 μg/ml of puromycin was added. A small sample of transduced and untransduced cells were analysed by flow cytometry to measure BFP expression and confirm successful transduction (Section 7.9). After a further 4 days, cells were passaged again, maintaining $12x10^6$ cells. As before, some cells were taken for analysis by flow cytometry (Section 7.9). On day 9, cells were detached, counted, centrifuged at 300g for 5 minutes then diluted in PBS. In parallel, aliquots of $5.5x10^6$ (500x) cells were pelleted and frozen at -80°C.

7.17.4.2 *In vivo* phase

Seventy wildtype (C57BL6/NTac) female mice aged 6-8 weeks were intravenously administered (via the tail vein) 100 μl of cells in PBS at a concentration of $5.5x10^6$ cells/ml. Mice were then Schedule 1 sacrificed at two timepoints: 4 hours post-dosing and day 19 post-dosing (35 mice per timepoint), and saline cardiac perfused (to remove any blood from the lungs, thus removing circulating tumour cells). The lungs were then collected and snap-frozen.

7.17.5 Processing of CRISPRa screen samples

gDNA was extracted from both the lung samples and the ‘day 0’ B16-F0-dCas9 cells using the Puregene kit (Qiagen) according to the manufacturer’s protocol. PCR reactions were performed with 500 ng of gDNA per reaction, using the Phusion® High-Fidelity PCR Master Mix with HF Buffer (#M0531L, NEB) to amplify the gRNAs. The forward primer contained an 8mer barcode, 5’ Illumina adapters and homology to the CRISPRia-v2 plasmid. The reverse primer contained 3’ Illumina adapters and homology to the CRISPRia-v2 plasmid. For each lung, 16 PCR reactions were performed and all products were pooled. A portion of this was purified to select for only the ~280 bp product, using a Select-a-Size DNA Clean & Concentrator kit according to the manufacturer’s protocol (#D4080, Zymo). PCR was also performed on gDNA from the B16-F0-dCas9 cells collected on the day of dosing and the library plasmid DNA. Purity of all PCR samples was confirmed by analysis on a Bioanalyser. The samples were then

combined in two pools (each containing 35 lung samples + 2 cell samples + 1 plasmid sample) and sequenced on a HiSeq2500. Two sequencing primers were used: a bespoke primer and a standard Illumina primer.

7.17.6 Analysis of sequencing data

All sequencing results were processed and further analysed by Vivek Iyer using the methods described in Section 6.2.3.

7.17.7 Validation of screen hits

7.17.7.1 Preparation of single gRNA plasmids

Single gRNAs (detailed in Section 6.2.4) were individually cloned into the CRISPRia_v2 backbone, following the Weissman lab protocol (this can be accessed at https://weissmanlab.ucsf.edu/CRISPR/CRISPR.html). Plasmids were sent for Sanger sequencing (Eurofins) to confirm successful insertion of the gRNAs. The gRNA plasmids were packaged into lentiviruses (Section 7.8.1) and titred in B16-F0-dCas9 cells (Section 7.17.3).

7.17.7.2 *In vitro* phase

A total of $5x10^5$ cells were transduced with each lentivirus at an MOI of 0.3 and 8 μg/ml polybrene, in a total volume of 5.5 ml. Cells were seeded in a T25 flask. Untransduced cells were maintained in parallel as a control. Cells were processed, selected and analysed by flow cytometry in the same way as described for the screen (Section 7.17.4.1).

7.17.7.3 *In vivo* phase

On day 10 post-transduction, cells were detached, counted and diluted to $2x10^6$ cells/ml in PBS. Intravenous administration was performed via the tail vein with 100 μl of cell suspension ($2x10^5$ cells per mouse). In every cohort, 8-10 wildtype (C57BL/6NTac) mice aged 6-8 weeks were dosed per gRNA (both sexes were used for different cohorts, but same sex used within a cohort). On day 10 post-dosing, lungs were collected from all mice and metastases were counted by eye. The number of metastases present in mice carrying cells transduced with each gRNA was compared to those carrying the control gRNAs (NTC pool) in the same cohort. A Mann-Whitney test was performed to determine the significance of any differences observed.