Chapter 5

Discussion and future directions

5.1 Summary of findings

In this project, we performed genome-wide CRISPR/Cas9 knockout screens in a panel of isogenic iPSCs with the aim of identifying novel synthetic lethal partners of 15 tumour suppressor genes. We engineered a panel of iPSC lines that each had LOF in a single TSG, prepared a genome-wide gRNA library suitable for screening in these cells and optimised a protocol to do so (discussed in Chapter 2). We screened the parental iPSC line and 21 KO lines. Analysis of the data revealed that whilst known fitness genes could be identified, the outputs were highly variable, even in replicates of the same cell line (discussed in Chapter 3). It was evident that this variability was likely caused by Cas9-induced toxicity, as a result of DSB formation. Despite this, we performed further analysis to identify candidate synthetic lethal partners of each TSG. Focusing on four TSGs that were members of the PBAF/BAF complex, we then attempted to experimentally validate hits from the screens (discussed in Chapter 4). Due to experimental issues, we were unable to complete validation in iPSCs but preliminary results in an independent cancer cell line highlighted several candidates for further study. We also analysed published cancer cell line screen datasets to identify potential SLIs involving the PBAF/BAF genes but found no novel significant associations.

5.2 CRISPR/Cas9 screening in iPSCs

5.2.1 Comparison with published stem cell screens

In the last 18 months, three groups have published genome-wide screens in hESCs,[238-240] but no iPSC screens have been published. Due to the lack of available literature at the start of this project, we did not anticipate the challenges that arose during iPSC screening. However, it is evident from these recent publications that the issues we faced were not specific to this project. In each of these studies, screens were performed with different lentiviral genome-wide gRNA libraries.[238-240] There were various other experimental differences which may influence the comparability of these studies, such as the substrate used to culture the cells, the Cas9 system and the library coverage (Table 5.1). Mair *et al.* (2019) repeated screening in the same cells using two different culture systems (mouse embryonic fibroblast feeder cells (MEFs) and laminin) and found that the results differed considerably. This highlights the fact that culture conditions can influence the identification of genes required for cell fitness, emphasising the need for cross-validation of results from CRISPR/Cas9 screens.

Table 5.1. Comparison of stem cell screens.

Study	Cell line	Culture substrate	Cas9 system	Target library coverage
This project	BOB, human iPSC line	Vitronectin	Constitutive Cas9	~150x
Yilmaz *et al.* (2018)	h-pES10, haploid hESC line	MEFs	Delivered in same vector as gRNAs	~700x
Mair *et al.* (2019)	H1, hESC line	MEFs and laminin	Inducible Cas9	~400
Ihry *et al.* (2019)	H1, hESC line	Vitronectin	Inducible Cas9	~100x

The outputs of these screens were analysed using similar but not directly comparable systems. To allow for comparison, Mair *et al.* (2019) re-processed the results from the other studies to match their analysis and calculated Bayes Factors for each gene. They assessed the overlap across all screens. Additionally, they considered the overlap with 1580 core fitness genes previously established by Hart *et al.* (2015) using CRISPR/Cas9 knockout screens in immortalised cell lines. Excluding these core fitness genes, a total of 36 genes were found to be essential for cell fitness in all of the stem cell screens. By cross-referencing these with genes that were significantly depleted in any of our three BOB screen replicates (based on BAGELR

analysis), 17 genes were common to our iPSC line and the hESC lines (Table B.1, Appendix B). As discussed in Section 3.3.1, BAGELR varies slightly from the original BAGEL and so re-processing of either our data or the published datasets may allow a more direct comparison and could increase this overlap. It is reassuring that we identified overlapping hits and our data may help in establishing a list of core stem cell fitness genes, which could be a useful reference for future studies. Colleagues at the WSI are performing genome-wide knockout screening in iPSCs from different human donors. They are using an alternative library using fewer gRNAs/gene and screening at a larger coverage than we did. It will be interesting to compare with their results, particularly as they may have significant variability between donors. This may also allow us to identify hits that are common to iPSCs but differ from ESCs.

5.2.2 Technical issues with screening stem cells

The largest issue we faced in our screens was Cas9-induced toxicity, which manifested in several ways. Vast cell death occurred in the days immediately following transduction, and there was a significant enrichment of NTC gRNAs (Section 3.2.2) and gRNAs targeting genes such as *TP53*, which play a role in the DNA damage response (Section 3.8). Another group recently published similar findings regarding the toxicity of Cas9 in a hESC line and explored this in more depth.[241] Using 47 gRNAs targeting 16 genes, they found that editing was highly efficient but the majority of cells did not survive. They selected a gRNA targeting a gene that was not expressed in the cell line and compared this with an NTC gRNA. The targeting gRNA caused a decrease in confluence over time, whereas cells expressing the NTC gRNA had increased confluence. They considered the possibility that this effect could be due to off-target activity, but found no evidence of editing at the top 6 predicted off-target sites. They also trialled transient Cas9 exposure and use of a Cas9 with enhanced specificity to reduce off-target activity, but toxicity was still observed.

In the same study, a high coverage (1000x) screen was performed using a library with 13,000 gRNAs, both in the presence and absence of Cas9 activity. In cells where Cas9 was not induced, the fold-changes for most gRNAs were evenly distributed, but they observed a 1.3-1.4 enrichment of NTC gRNAs when Cas9 was active. This suggested that there was a global depletion of targeting gRNAs. Mair *et al.* (2019) also observed toxicity when Cas9 was induced during screening, and noted an enrichment of non-targeting gRNAs. These data correlate well with the enrichment of NTC gRNAs observed in our screens (Fig. 3.2). Ihry *et*

al. (2018) analysed the fold-change of NTC gRNAs in screens of immortalised or tumour cell lines and found little enrichment, indicating that this toxicity effect is heightened in stem cells.

Ihry *et al*. (2018) demonstrated that CRISPR/Cas9 knockout did not affect expression of pluripotency markers, which supports data produced in this project (Section 2.8) and in a previous study.[273] Using RNA-seq analysis, they compared cells expressing a targeting gRNA vs an NTC gRNA and found that many genes involved in apoptosis were up-regulated in the DSB-induced cells. Interactome analysis indicated that p53 was linked to many of the expression changes associated with DSBs. They demonstrated that *TP53*-mutant cells increased in confluency despite DSB induction. However, the growth was ~50% less than in cells where Cas9 was not induced, suggesting that there was still an effect on cell fitness. In our screens, the NTC gRNAs were also enriched in a *TP53* KO line (Fig. 3.2), indicating that the toxicity effect was not solely dependent on the p53-mediated DNA damage response. However, we did find that the *TP53* KO screens were more reproducible than the parental (Section 3.6.1), had the highest recall of known core fitness genes (Section 3.4.3) and showed greater separation of established essential and nonessential genes (Section 3.4.2).

Similar to our findings, all of the published genome-wide screen studies in hESCs observed enrichment of *TP53*-targeting gRNAs.[238-240] Other genes were also identified, including *PMAIP1* and *CHEK2*, which we found to be recurrently enriched in our iPSC screens (Fig. 3.12). Ihry *et al.* (2019) performed further analysis of *PMAIP1*, a pro-apoptotic regulator downstream of p53, and found that this enrichment appeared to be specific to stem cells. Experimental validation confirmed that mutation of *PMAIP1* reduced Cas9-induced toxicity.[238]

5.2.3 Potential improvements to screening in iPSCs

Our findings and those of the few other related studies have been informative with regards to the ways in which stem cell screening protocols could be improved in future. Data regarding gene enrichment provides a better insight into the toxic response observed in stem cells and could provide strategies for improving the application of CRISPR/Cas9 in these cells. For example, transient inhibition of these DNA damage response pathways could help improve survival. However, even temporary interference with these genes would likely alter the results of any screen and would need to be considered when interpreting any results. Another key revelation is that NTC gRNAs are not an ideal control, which may also be relevant for other cell types. As an alternative, safe-targeting gRNAs could be used. These target genomic regions which have no functional impact, but still induce DSBs and so would act as a better control.

One option to improve the quality of our iPSC data would be to increase the coverage (i.e. transduce and maintain more cells per gRNA), potentially making it easier to differentiate between general depletion due to Cas9 toxicity and specific depletion due to gene essentiality. As discussed in Section 5.2.1, our initial coverage was much lower than others have used. The scale of our screening protocol was largely dictated by G418 selection, as the antibiotic did not work effectively when transduced cells were seeded densely. Untransduced iPSCs were highly sensitive to G418 selection regardless of density, but we found that the presence of resistant cells in the population impaired the selection capability. Cells were seeded sparsely to allow selection to occur and for cells to proliferate for more than a couple of days post-transduction. Due to the issues with measuring BFP expression (Section 2.7.3), it was difficult to confirm that selection was complete and if so, when this was achieved. However, there appeared to be no significant cell death after 1 week. In comparison to puromycin, G418 was much less efficient. Previous work in our lab (not described here) has shown that puromycin can effectively select these cells within 48 hours, at a density 4-fold higher than that used in our screens. Thus, it is not surprising that the majority of available gRNA library backbones have a puromycin selectable marker.

We were unable to use a puromycin-resistant library backbone because the KO iPSC lines were engineered to carry a puromycin cassette (Section 2.3). The cells were also resistant to blasticidin, which was used as a selectable marker for Cas9 expression (Section 2.6). In addition to G418, hygromycin B and zeocin antibiotics can also be used for selection of mammalian cells. Based on past experience, colleagues informed us that zeocin was difficult to use. Clara Alsinet had previously tested hygromycin B in the BOB iPSC line (data not shown) and found that G418 worked better, hence our decision to use this antibiotic. The cost of screening in iPSCs was significant due to the requirement for vitronectin coating on all culture dishes and daily medium changes. Performing screens at this scale also required a lot of time and incubator space. Thus, increasing the scale of the screen to improve library coverage was not feasible. However, if we had used puromycin selection, approximately 4-fold greater library coverage could have been achieved with more cells seeded per dish. In hindsight, using an alternative selectable marker for the KO iPSCs would have been preferable but engineering of these lines began before the screening aspect of this project was planned.

Another option to address the toxicity issue could be to screen with CRISPRi technology, which inhibits gene expression by fusing repression domains to a catalytically inactive Cas9. This would avoid the complication of DSB-induced toxicity as Cas9 would not cut the DNA. In theory, this should improve the quality of screen data by removing noise associated with

Cas9 toxicity. A higher library coverage could be achieved without the necessity to scale up to account for cell death, saving both time and money. One potential limitation of CRISPRi is that transcriptional repression may be less effective at reducing protein expression than a loss-of-function mutation. Mandegar *et al.* (2016) compared CRISPRi with CRISPR knockout in iPSCs using gRNAs targeting *OCT4* and *NANOG*. They found that CRISPRi was in fact more efficient, with almost complete loss of protein expression, whereas approximately a third of cells maintained expression using CRISPR knockout. Sequencing analysis highlighted that 30-50% of these cells had in-frame indels, which would explain the lack of protein knockout. Various others have shown successful application of CRISPRi in iPSCs and iPS-derived cells.[274-279] However, these studies have involved either targeted genes or more focused screens, not on a genome-scale. In future, a genome-wide CRISPRi screen could be performed in the parental BOB line to compare the results with our knockout screens.

Despite the challenges we faced, in some of our screens we were able to detect known essential genes and identify genes that were identified as being specific to stem cells in published studies. However, considering our aim was to identify specific genetic changes between isogenic cell lines, the variability we observed was a significant limitation. The potential improvements discussed here may improve the reproducibility of iPSC screens and allow more reliable identification of genetic interactions.

5.3 Screening for PBAF/BAF dependencies

To date, research into targeting PBAF/BAF mutant cancers has been largely focused on the most commonly mutated subunit, *ARID1A*. None of the previously reported dependencies associated with *ARID1A* loss were identified in our iPSC screens. This could be due to the quality of the screen data, as the *ARID1A* KO screens did not perform as well as others (Section 3.4). However, it could also be due to context-specificity of the previously identified synthetic lethal interactions. The most widely validated synthetic lethal partner of *ARID1A* is *ARID1B.* As we did not detect this in our screens, we performed further experiments to test this but found no evidence of an interaction (Section 4.2.1). More extensive validation could be performed, using more gRNAs or possibly using si/shRNA technology, however it is possible that this interaction does not occur in the BOB cell line. Many of the other *ARID1A* dependencies were found in OCCC cell lines and were not broadly validated in other tissue types, therefore it is possible that they are also specific to certain cell lineages. No studies have demonstrated systematic screening for synthetic lethality in *ARID1B, ARID2* or *PBRM1* mutants so we had little reference for comparing the output of our screens. We analysed large-scale pan-cancer CRISPR/Cas9 screen datasets but found no dependencies associated with LOF mutation in any of these 3 genes, although this may be due to the small number of mutants (Section 4.6.2).

In our screens, we identified candidate SLIs for all 4 PBAF/BAF subunits and reassuringly, some hits were identified in two independent KO clones for the same gene (Section 4.6.1). Due to the variability of our data and incomplete validation, it is difficult to draw any conclusions regarding these. Further experiments are required to confirm that these interactions occur in the iPSCs, and then these must be assessed in cancer cell lines to confirm they are not stem cell-specific. We chose to validate in a haploid CML line (HAP1) and had potentially promising preliminary data for some hits (Section 4.5) but repetition of this assay and further validation is needed. For clinical relevance, it would be ideal to instead focus on cell lines that represent the tissues most commonly affected by loss of these genes e.g. ovarian cancer for *ARID1A* and renal cancer for *PBRM1*. One possible strategy would be to harness the available CRISPR/Cas9 screen data to select cell lines for validation. Hits could be cross-referenced with screen data from PBAF/BAF mutant cancer lines. This may give an indication of whether a hit would validate without having to test a large panel of cancer cell lines. Although the small sample size could be an issue here, and it must be kept in mind that false negatives can occur in these screens so some hits may be missed.

5.4 Isogenic models for SLI screening

Choosing the right context to identify and validate candidate SLIs is one of the biggest challenges of not only this project, but synthetic lethality studies in general. In this project we chose iPSCs as a model as they have a clean genetic background and should therefore have limited confounding factors. Considering the limitations discussed in Section 5.2, further validation experiments are needed to assess how effective they were as a model. However, I expect that although technical improvements may be required, screening in iPSCs can be used to identify novel genetic interactions with higher confidence than in a cancer cell line with many genetic aberrations. The main barrier may not be the model, but rather that screening in any single cellular context is not sufficient to identify broadly applicable SLIs.

Analysis we performed on pan-cancer CRISPR/Cas9 screen datasets supported previous findings that *ARID1A* mutant cells are dependent on *ARID1B* (Section 4.2.2). However, it is clear from these analyses that, even though this was the strongest association identified in *ARID1A* mutants, this interaction is not fully penetrant. In this context, penetrance refers to the fraction of tumour cell lines harbouring a genetic mutation that are sensitive to inhibition of a synthetic lethal partner.[62] In the Sanger screen dataset, only 15% of *ARID1A* mutant lines were dependent on *ARID1B*; this was slightly higher at 30% in the Broad dataset (Fig. 4.5). This is not a unique observation; many SLIs have incomplete penetrance, and this is likely one of the main reasons that only one interaction has progressed to the clinic.[62] For example, several large-scale screens have been performed to identify synthetic lethal partners of *KRAS,* but few hits have replicated across multiple studies (as reviewed by Downward, 2015).[280] It is possible that the lack of reproducibility is due to technical issues, but even studies using the same screening method in different cell lines have shown a high degree of cell line-specificity for SLIs.[129] It is likely that many SLIs are either cell-type specific or specific to a certain genetic context, with other genes interfering with the interaction.

Screening across a panel of cancer cell lines and associating results with genetic mutations has evidently been useful in identifying SLIs such as the *ARID1A/ARID1B* pairing. However, this is limited by the number of cell lines available with existing mutations. The large genetic heterogeneity also makes it difficult to confidently deduce which, if any, single genetic change is responsible for the effect observed across all lines. The results in an isogenic system can be interpreted more clearly. Going forward, I would propose that an alternative strategy could be to combine both strategies by screening across multiple isogenic pairs. I would select cell lines that have naturally occurring LOF mutations in the PBAF/BAF genes but are

otherwise genetically heterogenous. CRISPR/Cas9 could then be used to correct the mutation and engineer a wildtype derivative. This strategy would allow robust identification of SLIs that are not specific to one cell line or tissue type, which is vital for any potential targets to be widely applicable in the clinic.

5.5 Future perspectives

In this project we produced a large amount of iPS cell screen data; screening in this cell type has not been shown in the literature and there are very few datasets from other pluripotent cells. Our data has given an insight into the significant variability that occurs when screening this cell type. This has been valuable in emphasising how important it is to replicate and validate screens thoroughly. In Section 5.2.3 I proposed several ways to address the issues we faced and I would recommend that increasing library coverage and/or using a technology such as CRISPRi would be the best approaches to deal with the issue of cell death. It was also evident that non-targeting gRNAs were poor controls as they did not induce a DNA damage response and were positively selected. For future screens, safe-targeting gRNAs which cut DNA may be a better control. We tested the pluripotency of the cells post-screen but in hindsight, it would have been valuable to measure expression of pluripotency markers during the screen whilst the cells were stressed and undergoing morphological changes. Our aim was to use this as a model rather than to understand the biology of the stem cells, but validating the state of the cells would be particularly pertinent for researchers interested in using screen data to study iPSCs. We did not have the opportunity to screen another iPSC line for comparison, but another group at WSI is screening multiple lines using a higher coverage and an independent library. We hope that integration of these datasets will provide a starting point for identifying core and patient-specific genes that are essential for iPS cell fitness.

Further validation is needed to determine the utility of our data for identifying synthetic lethal interactions. Whilst there may be true interactions present in the results, the variability makes the data difficult to interpret with confidence. It would have been valuable to do additional biological replicates of the knockout lines, as we did with the parental line. We did not detect known interactions with the PBAF/BAF genes but as these were not tested independently in our cell line, we could not conclude that this was a fault of the screen. We performed experiments to test the *ARID1A/ARID1B* interaction but going forward I would repeat this and test other published interactions using other methods e.g. siRNA and chemical inhibitors. Confirming the presence or absence of these interactions in the BOB cell line would

be a good indicator of screen performance. For future validation, I think it would be most valuable to test our candidate hits in cancer cell lines that have existing mutations in the gene of interest e.g. *ARID1A*-mutant ovarian cancer cell lines. Although HAP1 has technical advantages, it does not reflect the clinical setting that these interactions occur in. As a haploid cell line, it also removed our ability to identify interactions with haploinsufficient genes.

When it became clear that CRISPR/Cas9 KO screening in iPSCs would be challenging and the initial data was variable, I also became involved in another project in the Adams' lab. In doing so, I gained experience using a different screening technology in a different model system. I performed these experiments in parallel with my own project, whilst I analysed my screen data and carried out validation assays. In Chapter 6 I have detailed the background to this additional project, the results we obtained and the subsequent work that is ongoing.