

Identification of novel regulators of B lymphocyte biology

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Abstract

The differentiation of B cells into antibody secreting cells (ASCs) and the production of protective antibodies is a critical part of the adaptive immune response to infection. ASCs are also important for the formation of immunological memory which provides protection against reinfection and the generation of ASCs is the goal for almost all current vaccination strategies. Despite the importance of these cells, we still lack a complete understanding of the factors that control B cell differentiation into ASCs, ASC survival and antibody secretion, all of which must be tightly regulated to ensure an optimal immune response.

Here, I have developed a CRISPR/Cas9 mediated arrayed screening approach for the identification of novel positive and negative regulators of primary mouse B cell proliferation, survival, differentiation into ASCs and antibody secretion. By interrogating multiple gene sets I have identified all elements within the ASC gene signature that are essential for the *in vitro* generation of ASCs. I have also identified several novel negative regulators of the B cell differentiation process (*AB124611*, *Arhgef18*, *A430078G23Rik*, *Fam43a*, *Pold1*, *Ripk3*, *Rnf130* and *Rps6ka5*). This work has also uncovered a novel role for 6 genes, (*Cdv3*, *Hspa5*, *Sec61a1*, *Selk*, *Sumo2*, *Vcp*) in driving the proliferation of B cells. One of these genes, *Cdv3*, has no previous association with proliferation in any cell type and presents an exciting new candidate for further investigation.

I have demonstrated that within the ASC gene signature there are 35 genes which are essential for efficient antibody secretion. Interestingly, many of these genes are components of the ER protein processing pathway, however, not all elements of this pathway appear to be essential for antibody secretion. These results raise the possibility of there being a specific pathway for antibody secretion, or that the genes identified in this thesis may represent weak links in the ER protein processing pathway which could potentially be exploited therapeutically to inhibit antibody secretion in disease settings.

Finally, I have used an *Irf4* deficient mouse model to uncover a novel role for *Irf4* in the development of the peritoneal B-1a population. I have shown that *Irf4*^{-/-} mice lack peritoneal B-1a cells and by examining multiple stages of B-1a cell development I have demonstrated that in the absence of *Irf4*, B-1a cell development is blocked at the transitional B-1a stage. By employing RNA sequencing to analyse the transcriptional profiles of the remaining *Irf4*^{-/-} B-1 cells and analysis of previously published ChIP sequencing data, I have revealed a potential role for *Irf4* in directly activating the expression of *Bhlhe41*, a transcription factor that is required for B-1a cell development and homeostasis.

Together, the results from this thesis build upon decades of previous work on the genetic regulation of B cell biology. Integrating the novel regulators of B cell proliferation and differentiation that I have identified in this thesis into the current model of ASC generation will improve our understanding of how the decision between undergoing differentiation or maintaining the B cell fate is made. A detailed understanding of how this fate decision is made has far reaching implications for human health and disease as this information can be used to inform vaccine design, reveal the causes of immunodeficiencies or highlight novel avenues for targeting pathogenic ASCs in autoimmunity and cancer.

Declaration

This is to certify that:

- i. The thesis comprises only my original work towards PhD except where indicated in the Preface;
- ii. Due acknowledgement has been made in the text to all other material used;
- iii. The thesis is fewer than 100 000 words in length, exclusive of tables, bibliographies and appendices.

Stephanie Elise Trezise

October 2020

Preface

Pursuant to the regulations governing the degree of Doctor of Philosophy at the University of Melbourne, I hereby certify that:

- i. My contribution to chapter 5 was 90%. Analysis of the RNA sequencing data, including the generation of the MDS plot (Figure 5.5), heatmap (Figure 5.6a), gene ontology pathway plots (Figure 5.7) and volcano plot (Figure 5.8a) was performed by Isabella Kong.

Therefore, the author's total contribution to the work described in this thesis was approximately 95%.

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Abbreviations

ABTS	2,2'-azinobis-(3-ethylbenzthiazoline sulfonic acid)
AEC	3-amino-9-ethylcarbazole
AID	Activation-induced cytidine deaminase
APC	Allophycocyanin
ASC	Antibody secreting cell
Bach2	BTB domain and CNC homolog 2
Bcl6	B cell lymphoma 6
BCR	B cell receptor
BFP	Blue fluorescent protein
Blimp-1	B lymphocyte induced maturation protein 1
BSA	Bovine serum albumin
BV	Brilliant violet
CD	Cluster of differentiation
CD40L	CD40 ligand
ChIP	Chromatin immunoprecipitation
CRISPR	Cluster regular interspaced short palindromic repeats
crRNA	CRISPR activating ribonucleic acid
CSR	Class-switch recombination
CTY	Cell trace yellow
CVID	Common variable immunodeficiency
DEG	Differentially expressed genes
DMEM	Dulbecco's Modified Eagle Medium
DMF	<i>N,N</i> -Dimethylformamide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
dsDNA	Double stranded deoxyribonucleic acid

EDEM	Endoplasmic reticulum degradation-enhancing alpha-mannosidase like
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
fDC	Follicular Dendritic cell
FDR	False discover rate
FITC	Fluorescein isothiocyanate
FL	Foetal liver
FoB	Follicular B
FPKM	Fragments per kilobase of exon length per million mapped fragments
FVD	Fixable viability dye
GC	Germinal Centre
GEF	Guanine exchange factor
GFP	Green fluorescent protein
GO	Gene ontology
gRNA	Guide ribonucleic acid
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
Ig	Immunoglobulin
IL	Interleukin
Irf4	Interferon regulatory factor 4
KEGG	Kyoto Encyclopedia of Genes and Genomes

LPS	Lipopolysaccharide
LSL	Lox-stop-lox
MACS	Magnetic activated cell sorting
MAGeCK	Model-based analysis of genome-wide CRISPR-Cas9 knockout
MGI	Mouse Genome Informatics
MLE	Maximum likelihood estimation
mRNA	Messenger ribonucleic acid
MZ	Marginal zone
NEAA	MEM non-essential amino acids
NHEJ	Non-homologous end joining
Pax5	Paired box gene 5
PB	Pacific Blue
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-cyanine 7
PI	Propidium Iodide
PRC2	Polycomb repressive complex 2
Prdm1	PR domain zinc finger protein 1
QC	Quality control
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SHM	Somatic hypermutation
TCRb	T cell receptor beta
TD	T cell-dependent
Tfh	T follicular helper cell

TI	T cell-independent
TLR	Toll-like receptor
Tr-B1a	Transitional B-1a
tracrRNA	Trans-activating CRISPR RNA
UPR	Unfolded protein response
VISPR	Visualization for CRISPR
WEHI	The Walter and Eliza Hall Institute of Medical Research
WT	Wildtype
Xbp1	X-box protein 1

Table of contents

Declaration	iv
Preface	v
Acknowledgement	vi
Abbreviations	viii
Chapter 1. Literature Review	1
1.1. The adaptive immune response	2
1.2. The B lymphocyte lineage	2
1.2.1. B cell development	2
1.2.2. Mature B cell subsets	3
1.2.3. Germinal centre B cells	4
1.2.4. Memory B cells	4
1.2.5. Antibody secreting cells	5
1.3. Activation of naïve B cells	8
1.4. Known negative regulators of B cell differentiation	9
1.4.1. Paired box gene 5	9
1.4.2. BTB and CNC homology 2	11
1.4.3. B cell lymphoma 6	11
1.4.4. Ets1	12
1.4.5. Other genes implicated in the negative regulation of B cell differentiation	13
1.5. Known positive regulators of B cell differentiation	14
1.5.1. Interferon regulatory factor 4	14
1.5.2. B lymphocyte induced maturation protein 1	17
1.5.3. X-box binding protein 1	18
1.5.4. Other known positive regulators of ASC differentiation	19
1.6. Protein homeostasis in antibody secreting cells	19
1.6.1. The ER-associated degradation pathway	19
1.6.2. The mammalian unfolded protein response	20
1.6.3. The unfolded protein response in antibody secreting cells	22
1.7. The antibody secreting cell gene signature	24
1.8. CRISPR/Cas9 genome editing	24
1.9. Thesis aims	25

Chapter 2. Materials and methods	27
2.1. Materials	28
2.2. Methods	54
2.2.1. Mice	54
2.2.2. B cell isolation and culture	54
2.2.3. Flow cytometry and cell sorting	54
2.2.4. Enzyme-linked immunosorbent assay (ELISA)	55
2.2.5. Enzyme-linked immunospot (ELISpot)	55
2.2.6. CRISPR gRNA libraries	56
2.2.7. Production of lentiviral vectors	56
2.2.8. Transduction of primary B cells	56
2.2.9. Irradiation and fetal liver reconstitutions	57
2.2.10. RNA isolation	57
2.2.11. RNA library preparation and sequencing analysis	57
2.2.12. gDNA isolation	58
2.2.13. DNA library preparation and sequencing analysis	58
 Chapter 3. Identification of positive regulators of B cell differentiation, proliferation, and antibody secretion	 59
3.1. Introduction	60
3.2. Results	63
3.2.1. Development of an arrayed targeted CRISPR screen for primary B cells	63
3.2.2. A targeted CRISPR screen reveals the essential regulators of ASC differentiation	69
3.2.3. A targeted CRISPR screen reveals regulators of B cell survival and proliferation	78
3.2.4. A targeted CRISPR screen reveals regulators of LPS driven antibody secretion	82
3.3. Discussion	87
3.3.1. Few elements within the ASC gene signature are essential for B cell differentiation <i>in vitro</i> .	87
3.3.2. Novel regulators of B cell proliferation and survival	89
3.3.3. Identification of genes that are essential for antibody secretion	91
3.3.4. Future directions	94

Chapter 4. Identification of novel negative regulators of antibody secreting cell differentiation	96
4.1. Introduction	97
4.2. Results	100
4.2.1. Design and optimization of a genome wide CRISPR/Cas9 screen to identify novel negative regulators of B cell differentiation	100
4.2.2. Analysis of whole genome screen results using MAGeCK-Flute	104
4.2.3. Design of an arrayed targeted CRISPR/Cas9 screen to identify novel negative regulators of B cell differentiation	115
4.3. Discussion	118
4.3.1. A genome wide pooled CRISPR/Cas9 screen failed to identify novel regulators of B cell differentiation	118
4.3.2. A targeted arrayed CRISPR/Cas9 screen reveals novel negative regulators of B cell differentiation	121
 Chapter 5. <i>Irf4</i> is essential for B-1a cell development through the activation of the transcription factor <i>Bhlhe41</i>	 127
5.1. Introduction	128
5.2. Results	132
5.2.1. <i>Irf4</i> ^{-/-} mice lack peritoneal B-1a cells	132
5.2.2. The absence of B-1a cells in <i>Irf4</i> ^{-/-} mice is cell intrinsic	137
5.2.3. <i>Irf4</i> is required for B-1a cell development	137
5.2.4. CD5 ⁻ <i>Irf4</i> ^{-/-} B1 cells are true B-1b cells	138
5.2.5. <i>Irf4</i> regulates expression of <i>Bhlhe41</i> , a transcription factor that is essential for B-1a development and function	144
5.2.6. <i>Irf4</i> deficient B1 cells have altered BCR signaling and proliferation	148
5.3. Discussion	150
 Chapter 6. Future directions and conclusions	 154
6.1. Future work	155
6.2. Potential implications	157
6.3. Conclusions	161
 Bibliography	 162

Chapter 1. Literature Review

1.1. The adaptive immune response

The immune system is a complex network of many cell types that is responsible for protecting the body from pathogens. The mammalian immune response can be divided into two arms: innate and adaptive. The innate immune system mounts a rapid but relatively non-specific response against an infection. Alternatively, the adaptive immune system is highly specialized and has the ability to distinguish between specific pathogens. This response is orchestrated by two cell lineages, T lymphocytes and B lymphocytes, with each cell expressing antigen receptors with a unique antigen specificity. A hallmark of the adaptive immune response is the ability to protect the host from reinfection with the same pathogen due to the generation of an immunological memory, either from the presence of antigen specific memory cells or long-lived antibody secreting cells (ASCs). ASC are the producers of antibodies, the key effector proteins of the humoral immune response.

1.2. The B lymphocyte lineage

1.2.1. B cell development

B cells are generated from hematopoietic stem cells (HSCs) and must progress through multiple progenitor stages before they are able to respond to antigens. During development, B cells will physically rearrange the genes that encode the immunoglobulin (Ig) heavy and light chains (Chi et al., 2020). Ig genes contain a number of variable (V), diversity (D) and joining (J) gene segments and after rearrangement B cells will express a B cell receptor (BCR) with one V, D, and J, and one V and J segment in the heavy and light chain respectively. Collectively, B cells have a huge BCR repertoire due to diversity in VDJ gene segment combinations. This repertoire is further diversified by the addition of non-templated nucleotides by terminal deoxynucleotidyl transferase during the recombination process. Following VDJ recombination, immature B cells can migrate to the spleen where they can undergo maturation.

1.2.2. Mature B cell subsets

In mice there are three subsets of mature B cells, Follicular B (FoB), Marginal Zone (MZ) and B1 B cells. While all three populations express a membrane bound BCR, they differ in their physical location, surface phenotype and antigen responsiveness. FoB cells are the predominant B cell population and reside within B cell follicles in the spleen and lymph nodes. These cells are identified by their surface expression of IgM^{low}IgD^{high}CD23^{high}CD21^{int}.

MZ B cells also reside in the spleen but only constitute 5-10% of the splenic B population and are located in the marginal sinus (Cerutti et al., 2013). This positioning means that they ideally located to be in contact with blood-borne antigens. MZ and FoB cells arise from the same transitional B cell population, and the strength of BCR signalling determines which cell type the transitional B cells will develop into with weaker BCR signalling promoting development in MZ B cells and strong BCR signalling driving FoB development (Pillai and Cariappa, 2009). In contrast to FoB cells, MZ B cells are IgM^{high}IgD^{low}CD23^{low}CD21^{hi} and mount more rapid responses to activation (Oliver et al., 1997; Oliver et al., 1999). This rapid activation, in combination with their location within the spleen, has led to the view that these cells are specialized to form the early response to blood-borne pathogens.

The third mature B cell subset is B1 cells, which are considered to more innate-like than FoB or MZ B cells. B1 cells constitute a very small proportion of the splenic B cell population, however, they are the predominant B cell subtype found in the peritoneal and pleural cavities (Hayakawa et al., 1985; Hayakawa et al., 1983). B1 cells are identified by the surface phenotype of B220^{+/lo}CD23⁻IgD^{lo}IgM^{hi} and can be further subdivided into CD5⁺ B-1a cells, and CD5⁻ B-1b cells (Stall et al., 1992). Unlike FoB and MZ B cells, B1 cells have a restricted BCR repertoire and a proportion of B-1a cells express BCRs that recognize self-antigens (Hayakawa et al., 1990; Hayakawa et al., 1984; Pennell et al., 1989a; Pennell et al., 1989b). B1 cells are the predominant source of “natural” antibodies, which are low-affinity polyreactive antibodies that are secreted in the absence of exogenous antigen stimulation (Hayakawa et al., 1983; Smith and Baumgarth, 2019). In contrast to the MZ and FoB populations, which are

constantly replenished from bone marrow HSCs, B1 cells are predominantly generated from foetal HSCs and are maintained through a process of self-renewal (Hayakawa et al., 1985; Hayakawa et al., 1986).

1.2.3. Germinal centre B cells

Following activation, B cells will undergo a proliferative burst and can form a highly specialized structure known as a germinal centre (GC) (Suan et al., 2017). As GCs increase in size, they will polarize into two zones, the dark zone containing rapidly proliferating centroblasts, and the light zone containing non-dividing centrocytes. Centrocytes will interact with other specialized GC cells including follicular dendritic cells (fDCs) and follicular T (Tfh) cells (Vinuesa et al., 2016). These cells will provide help to the B cells through antigen presentation, CD40-CD40L interactions and cytokine secretion, and will facilitate the process of affinity maturation. B cells that have received help will then migrate to the dark zone and undergo proliferation (Victora et al., 2010). Proliferating centroblasts can undergo a process called somatic hypermutation (SHM) (Chi et al., 2020). AID deaminates cytosines in the Ig variable regions, converting them to uracil and creating uracil-guanine mismatches. DNA mismatches are then repaired by cellular DNA repair pathways in a way that introduces mutations into the Ig variable regions, altering BCR-antigen affinity. These cells will then return to the light zone and compete for antigen binding and survival signals (Victora et al., 2010). After multiple rounds of SHM and proliferation, the GC B cells that express BCRs with the highest affinity for antigen will be selected to differentiate into ASCs (Phan et al., 2006). This process also results in the generation of memory B cells, and it is thought that GC B cells with a lower affinity for antigen are selected into this compartment (Shinnakasu et al., 2016).

1.2.4. Memory B cells

Memory B cells are an important component of immunity and are essential for the rapid increase in pathogen-specific antibody production following a second exposure during reinfection (Kurosaki et al., 2015). Memory B cells are phenotypically and transcriptionally more similar to naïve B cells than to GC B

cells or ASCs, however, there are key differences between the naïve and memory populations (Bhattacharya et al., 2007). Memory B cells are an antigen experienced population and have usually, but not always, gone through the processes of class-switch recombination (CSR) and affinity maturation (Kurosaki et al., 2015; Suan et al., 2017). In comparison with naïve FoB cells, memory B cells mount a more rapid proliferative response to stimulation and have an increased rate of differentiation (Tangye et al., 2003a; Tangye et al., 2003b). Following reactivation, memory B cells can either differentiate into ASCs or can initiate a GC response and undergo SHM to further increase their antigen affinity (Kurosaki et al., 2015).

1.2.5. Antibody secreting cells

ASCs are the terminally differentiated cells of the B lymphocyte lineage. While B cells constitute a large proportion of cells within lymphoid organs, ASCs are a rare population that typically make up less than 1 % of cells within the spleen and bone marrow of adult mice. During the differentiation process, these cells undergo extensive transcriptional and morphological changes, including a massive expansion of their endoplasmic reticulum (ER), which facilitates their sustained high levels of antibody production. In contrast to B cell subsets, which express a membrane bound BCR, ASCs express a secretory form known as antibody. This switch is due to ASCs utilizing an alternative polyadenylation site in the *Ig* heavy chains, resulting in the production of shorter transcripts that lack a transmembrane domain (Peterson, 2011). ASCs are highly specialized antibody producing cells, with approximately 70 % of their transcriptome being devoted to expression of *Ig* genes, compared to B cells where *Ig* transcripts only constitute around 2 % of the transcriptome (Shi et al., 2015). Reflective of this specialization, a single ASC is capable of secreting approximately 2 ng of antibody per day (Brinkmann and Heusser, 1993).

There are two subsets of ASCs, plasma cells and plasmablasts, which differ in their level of Blimp-1 expression, surface marker phenotype, proliferation and lifespan. The ASC compartment in the spleen consists of roughly equal numbers of plasmablasts and plasma cells, however, plasma cells are the dominant

population in the bone marrow. Plasmablasts are a short-lived, proliferating population that express intermediate levels of Blimp-1 (Kallies et al., 2004). It is these characteristics, along with the observation that this population appears shortly after immunization, that has led to the view that plasmablasts are the more immature ASC population (Blink et al., 2005; Kallies et al., 2004). In contrast, plasma cells express high levels of Blimp-1, are non-dividing and are capable of living for years (Kallies et al., 2004; Manz et al., 1998; Slifka et al., 1998). The genetic network driving ASC generation and function is generally described as being under the control of three transcription factors: Irf4, *Prdm1*/Blimp-1 and Xbp1.

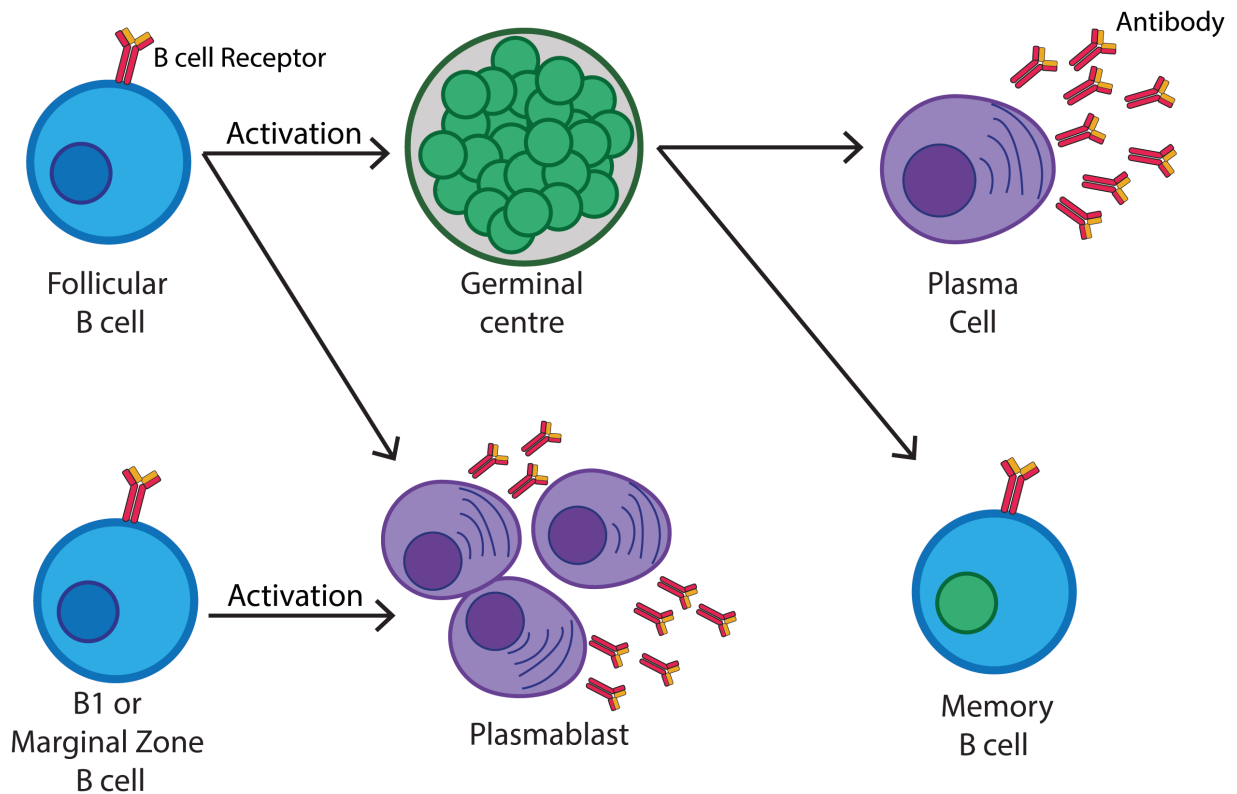


Figure 1.1 The differentiation of mature B cell subsets into ASCs

There are three subsets of mature B cells, follicular, marginal zone and B1, which occupy specialized niches within the body. All B cell subtypes express a B cell receptor. Once activated, B cells can proliferate and differentiate into plasmablasts. Plasmablasts are a population of short-lived, proliferating antibody secreting cells (ASCs). Follicular B cells also have the ability to form a germinal centre where they will undergo the process of affinity maturation. After exiting the germinal centre, these cells can become memory B cells or plasma cells. Memory B cells retain a B cell phenotype but display accelerated response kinetics to subsequent activation. Plasma cells are also a class of ASC, however, this population is long-lived and quiescent. Although there is evidence that B1 and marginal zone B cells can contribute to the long-lived plasma cell pool under certain conditions, this diagram represents the typical response mounted by each B cell subtype following activation.

1.3. Activation of naïve B cells

An effective humoral immune response requires naïve B cells to be activated, undergo multiple rounds of proliferation and to differentiate into GC B cells, memory B cells and ASCs. Naïve B cells can be activated by two classes of antigens, T cell dependent (TD) and T cell independent (TI). TD antigens are typically soluble proteins or protein-conjugated molecules that will also activate naïve CD4⁺ T cells. TI antigens are more diverse and are usually highly repetitive structures, such as lipopolysaccharides (LPS) from bacterial cell walls, that can be recognized by Toll like receptors (TLRs).

Upon activation, B cells undergo a burst of proliferation, resulting in a large pool of B cells that carry an identical BCR. Additionally, activated B cells can undergo CSR to alter the constant region domains of their BCR (Chi et al., 2020). Following this proliferative burst, activated B cells have the potential to differentiate into extrafollicular plasmablasts or form a GC. Extrafollicular plasmablasts produce relatively low-affinity antibodies, while ASCs that are derived from GCs, and have therefore undergone affinity maturation, produce high-affinity antibodies (MacLennan et al., 2003; Suan et al., 2017). The fate decisions that these cells make is influenced by the stimulation type, stimulation strength and the presence of additional factors such as T cell cytokines. Proliferation is intrinsically linked to B cell fate as the probability that cells will undergo CSR or differentiate increases with each cell division (Deenick et al., 1999; Hasbold et al., 2004; Hodgkin et al., 1996). Furthermore, B cells must divide several times before they are able to undergo either of these processes (Hodgkin et al., 1996; Scharer et al., 2020). The probability that a B cell will differentiate or undergo CSR can be modulated by cytokines. For example, IL-4 increases the proportion of cells in each division that undergo CSR and IL-5 increases the likelihood that cells will differentiate (Hasbold et al., 2004).

All three B cell subsets have different kinetics in their response to activation (Fairfax et al., 2007; Oliver et al., 1997; Oliver et al., 1999; Snapper et al., 1993). B1 cells display the most rapid differentiation responses, while FoB cells have the longest delay between activation and differentiation (Fairfax et al., 2007). FoB cells also show a delay in their proliferative responses to activation compared to

MZ and B1 B cells (Oliver et al., 1999). Each B cell type also has an inherent difference in their capacity to respond to different antigens, with B1 cells undergoing a robust differentiation response to the TLR9 agonist CpG, while FoB cells are unable to differentiate in response to CpG (Fairfax et al., 2007).

Many aspects of the B cell activation and differentiation process can be reproduced *in vitro*. TI responses can be induced with antigens including CpG and LPS, while TD activation can be mimicked by the ligation of CD40 on the B cell surface and by the addition of T cell cytokines including IL-4 and IL-5. While *in vitro* B cell activation doesn't fully recapitulate the *in vivo* response, it does provide a defined system to interrogate the regulation of the activation and differentiation process.

1.4. Known negative regulators of B cell differentiation

B cells and ASCs are regulated by two mutually antagonistic genetic networks, therefore, the differentiation process involves a fundamental change in the cells transcriptional program. If B cells differentiate before germinal centre formation or class-switch recombination can occur, the antibody response mounted will be sub-optimal, therefore, it is critical that the timing of the differentiation process is tightly controlled. Previous studies have identified a number of factors that negatively regulate the differentiation process. These factors primarily regulate this process by maintaining the expression of the B cell transcriptional program and by repressing the expression of plasma cell genes.

1.4.1. Paired box gene 5

Paired box gene 5 (Pax5) is a transcription factor that is expressed throughout B cell development and in all mature B cell subsets until it is downregulated in one of the earliest stages of differentiation (Barberis et al., 1990; Fuxa and Busslinger, 2007; Kallies et al., 2007). Expression of *Pax5* is critical for commitment to the B cell lineage and *Pax5* deficient mice lack B lymphocyte populations due to a block in B lymphopoiesis at the early pro-B cell stage (Nutt et al., 1997; Urbánek et al., 1994). These *Pax5*^{-/-} progenitors remain uncommitted to the B cell lineage and

can be induced to differentiate down alternate cell lineages (Nutt et al., 1999; Rolink et al., 1999). Furthermore, sustained *Pax5* expression is required for maintaining commitment to the B cell lineage as deletion of *Pax5* in mature B cells results in cells reverting to an earlier progenitor state, which can then be induced to develop into alternate immune cell types (Cobaleda et al., 2007; Mikkola et al., 2002). In addition to directly regulating gene expression, *Pax5* has been implicated in the creation and maintenance of B-lineage specific 3-dimensional chromatin architecture throughout B cell development (Johanson et al., 2018).

Pax5 regulates B cell function and identity by activating the expression of B cell genes while repressing the expression of lineage inappropriate genes. It is required for the induction of expression of genes involved in pre-BCR and BCR signal transduction including *Cd19*, *Cd21*, *Cd79a* and *Blnk* (Horcher et al., 2001; Nutt et al., 1998; Schebesta et al., 2002). Consequentially, *Pax5*^{-/-} B cells have impaired pre-BCR and BCR signalling (Nera et al., 2006; Thévenin et al., 1998). *Pax5* also induces the expression of other transcription factors with important roles in the B lymphocyte lineage including *Bach2*, *Irf4*, *Irf8*, *SpiB* and *Aiolos/Ikzf3* (Pridans et al., 2008; Schebesta et al., 2007).

Pax5 is downregulated during the earliest stages of differentiation, allowing the expression of non-B cell genes including the ASC surface markers *Cd28*, *Cd93* and *Cd138* (Delogu et al., 2006; Horcher et al., 2001; Kallies et al., 2007). *Pax5* likely acts as a negative regulator of B cell differentiation and it is thought that the downregulation of *Pax5* is an essential part of the differentiation process. In support of this notion, overexpression of *Pax5* in activated mouse B cells reduces the rate of B cell differentiation (Lin et al., 2002). Furthermore, mutation of *Pax5* in the chicken B cell line DT40 induces cells to upregulate the plasma cell genes *Prdm1* and *Xbp1*, and to differentiate into IgM secreting cells (Nera et al., 2006). However, recent work has challenged the idea that repression of *Pax5* is essential for B cell differentiation, showing that *Pax5* transgenic B cells, where *Pax5* expression is under the control of the *Igh* promoter and therefore is not downregulated during differentiation, display a largely normal differentiation response (Liu et al., 2020).

1.4.2. BTB and CNC homology 2

BTB and CNC homology 2 (*Bach2*) is a transcriptional repressor which is positively regulated by Pax5 and is expressed throughout B cell development until differentiation (Muto et al., 1998; Schebesta et al., 2007). Despite this expression pattern, B cell development can occur relatively unaffected by the absence of *Bach2* (Muto et al., 2004). *Bach2* has an important role in preventing B cell differentiation as it heterodimerises with Maf proteins and prevents the expression of *Prdm1* (Muto et al., 1998; Ochiai et al., 2006). Consequentially, *Bach2*^{-/-} B cells will differentiate more rapidly and to a greater extent than their WT counterparts (Muto et al., 2010). *Bach2* deficient B cells also have an inability to undergo CSR (Muto et al., 2010; Muto et al., 2004). In the absence of *Bach2*, *Prdm1* is prematurely expressed, resulting in the repression of *Aicda* expression and differentiation before CSR can occur (Muto et al., 2010). In addition to this, *Bach2*^{-/-} B cells have an impaired ability to form GCs following immunization and have a decreased ability to undergo affinity maturation compared to *Bach2*^{+/+} B cells (Muto et al., 2004).

1.4.3. B cell lymphoma 6

B cell lymphoma 6 (*Bcl6*) is a negative regulator of differentiation that plays an important role in the GC response. *Bcl6* is highly expressed in GC B cells and *Bcl6*^{-/-} mice are unable to form GCs or generate high-affinity antibodies (Cattoretti et al., 1995; Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997). *Bcl6* primarily acts as a transcriptional repressor which regulates transcription through interactions with co-repressors and facilitates SHM by repressing DNA damage responses (Huang and Melnick, 2015). *Bcl6* functions as a negative regulator of differentiation by cooperating with *Bach2* to inhibit the expression of *Prdm1* (Huang et al., 2014; Ochiai et al., 2008; Shaffer et al., 2000; Tunyaplin et al., 2004). *Bcl6* expression is downregulated GC B cells following CD40 signalling in an *Irf4*-dependent manner, allowing differentiation into ASCs (Saito et al., 2007). Interestingly, *Bcl6* also regulates GC formation in a B cell extrinsic manner as it is required for the development of Tfh cells (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009).

1.4.4. Ets1

Ets1 is a transcription factor that is highly expressed throughout B cell development until it is downregulated following B cell activation (Anderson et al., 1999; Luo et al., 2014). *Ets1* is required for the normal development of mature B cells, as *Ets1*^{-/-} mice have an accumulation of pro-B cells, lack MZ B cells, and their FoB cells have an altered expression of multiple B cell surface markers including IgD and CD23 (Bories et al., 1995; Eyquem et al., 2004; Wang et al., 2005). In addition to having altered surface marker expression, *Ets1*^{-/-} FoB cells have altered BCR and TLR signaling as they fail to upregulate markers of B cells activation after stimulation and have an impaired proliferative response (Bories et al., 1995; Eyquem et al., 2004). There have been conflicting reports as to whether *Ets1* deficient mice have a peritoneal B-1a population, therefore, a potential role for *Ets1* in B1 cell biology remains unclear (Eyquem et al., 2004; Wang et al., 2005). *Ets1* appears to be a negative regulator of both ASC differentiation and memory B cell formation *in vivo*, as *Ets1*^{-/-} mice have expanded ASC populations, increased serum antibody titers and an increased frequency of memory B cells (Barton et al., 1998; Bories et al., 1995; Luo et al., 2014; Sunshine et al., 2019). *Ets1*^{-/-} mice also have an increase in class-switched B cells, although this is not a B cell intrinsic effect (Sunshine et al., 2019).

Ets1^{-/-} B cells exhibit increased differentiation *in vitro* to a range of stimulation conditions (John et al., 2014; Saelee et al., 2017; Wang et al., 2005). Conversely, B cells overexpressing *Ets1* have a reduced rate of differentiation (John et al., 2014; John et al., 2008; Luo et al., 2014; Saelee et al., 2017). *Ets1* has dual roles in the inhibition of B cell differentiation. It is able to directly induce the expression of B cell genes, including *Pax5* and genes involved in B cell activation and signal transduction (John et al., 2014; Saelee et al., 2017). *Ets1*, however, is not the only gene responsible for activating and maintaining *Pax5* expression in mature B cells, as naïve *Ets1*^{-/-} B cells do not have decreased expression of *Pax5* (Saelee et al., 2017). *Ets1* also post-translationally regulates Blimp-1 activity by interacting with Blimp-1 and inhibiting its DNA binding ability (John et al., 2014; John et al., 2008).

1.4.5. Other genes implicated in the negative regulation of B cell differentiation
There are multiple other factors that have been implicated in negatively regulating ASC differentiation. *Id3* prevents the expression of *Prdm1* in activated B cells by negatively regulating the activity of E-proteins including E2A and E2-2 (Gloury et al., 2016). *Id3*^{-/-} B cells have an increased rate of differentiation, while B cells overexpressing *Id3* are unable to differentiate. *Mitf*^{-/-} B cells can spontaneously differentiate *in vitro* in the absence of stimulation and overexpression of *Mitf* prevents differentiation (Lin et al., 2004). c-Rel is a subunit of the canonical NF-κB pathway and inhibits differentiation by promoting the expression of *Bach2* (Roy et al., 2019). Following differentiation, Blimp-1 then directly represses c-Rel expression. Fra1, a member of the Fos family, negatively regulates expression of *Prdm1* by competing with another Fos protein, c-Fos, for binding in the *Prdm1* promotor (Grötsch et al., 2014).

There are also multiple known instances of transcription factors acting co-operatively to negatively regulate ASC differentiation. Irf8 and PU.1/*Spi1* bind together to composite ETS-IRF DNA motifs. *Irf8*^{-/-} or *Spi1*^{-/-} B cells only display slight alterations in rate of differentiation compared to WT B cells (Feng et al., 2011; Polli et al., 2005; Willis et al., 2017). However, there appears to be partial redundancy in the role of each of these genes as *Irf8*^{-/-} *Spi1*^{-/-} double deficient B cells display a dramatic increase in differentiation rate (Carotta et al., 2014). Similarly, *Spib*^{-/-} B cells have a slight increase in ASC differentiation and this is enhanced in *Spib*^{-/-} *Spi1*^{-/-} cells (Willis et al., 2017).

Several epigenetic regulators have also been implicated in regulating the rate of B cell differentiation. B cells lacking the DNA methyltransferase Dmmt3 have increased GC formation and ASC differentiation, suggesting the Dmmt3-dependent DNA methylation has a role in limiting both of these processes (Barwick et al., 2018). Interestingly, Ezh2 appears to have opposing roles in either repressing or promoting ASC differentiation at different stages of the differentiation process. Ezh2 is the catalytic subunit of the PRC2 complex which deposits the repressive histone mark H3K27me3 (Cao et al., 2002). *Ezh2*^{-/-} B cells have an impaired ability to differentiate, however, pharmacological inhibition

of Ezh2 activity in pre-activated B cells results in an increase in ASCs (Guo et al., 2018; Scharer et al., 2018).

1.5. Known positive regulators of B cell differentiation

The differentiation of B cells into ASCs is generally referred to as being regulated by a triad of transcription factors, Irf4, Blimp-1 and Xbp1. The differentiation and function of ASCs requires extinguishing the B cell genetic program and remodelling of the cell to have the capacity to produce massive amounts of secreted antibody. Consequentially, known positive regulators of ASC biology have been shown to directly repress the expression of B cell genes and/or drive the expansion of the ER and the unfolded protein response (UPR) to increase the cells protein production capacity.

1.5.1. Interferon regulatory factor 4

Interferon regulatory factor 4 (Irf4) is essential for many processes throughout the B cell lineage and is expressed in all B cell stages, with the exception of GC B cells (Cattoretti et al., 2006). Irf4 can function both as a transcriptional activator and repressor and its activity is often dependent on binding to various co-factors (De Silva et al., 2012). During B cell development, Irf4 acts redundantly with Irf8 to control the transition from proliferating pre-B cells to immature B cells (Lu et al., 2003). *Irf4*^{-/-} *Irf8*^{-/-} double deficient pre-B cells are unable to downregulate the pre-BCR, undergo Ig light chain rearrangement, and exit the cell cycle, resulting in a developmental block at this stage (Lu et al., 2003). Irf4 is required for the correct formation of the mature B cell compartment as Irf4 deficient mice have fewer FoB cells and an expanded MZ B cell population (Mittrücker et al., 1997; Simonetti et al., 2013; Willis et al., 2014). It also has a potential role in maintaining tolerance by promoting BCR editing in autoreactive immature B cells (Pathak et al., 2008).

Irf4 has a critical role in ASC differentiation, in part due to its role in promoting expression of *Prdm1* and repressing the expression of *Bcl6* (Klein et al., 2006; Kwon et al., 2009; Mittrücker et al., 1997; Ochiai et al., 2013; Saito et al., 2007;

Sciammas et al., 2006). *Irf4*^{-/-} B cells are unable to undergo differentiation, and mice that lack *Irf4* expression in the B cell lineage have a complete absence of ASCs (Klein et al., 2006; Mittrücker et al., 1997; Ochiai et al., 2013). *Irf4* functions in a dose-dependent manner as high expression of *Irf4* drives differentiation, while low expression of *Irf4* is critical for CSR (Sciammas et al., 2006). Some expression of *Irf4* is required for CSR as *Irf4* deficient B cells do not upregulate *Aicda*/AID and are consequentially unable to undergo isotype switching (Klein et al., 2006; Sciammas et al., 2006). Additionally, *Irf4* is required for GC formation both in a B cell intrinsic manner and due to its role in Tfh cell generation (Bollig et al., 2012; Kwon et al., 2009; Ochiai et al., 2013; Willis et al., 2014). After differentiation, *Irf4* is an essential factor for ASC survival as the *in vivo* deletion of *Irf4* in *Irf4*^{fl/-} *CreERT2*^{T/+} mice results in the rapid loss of the ASC compartment (Tellier et al., 2016). A recent study has demonstrated that this survival defect can be overcome by the expression of the pro-survival gene *Bcl2* (Low et al., 2019). Analysis of *Irf4*^{fl/-} *CreERT2*^{T/+} *Bcl2*-transgenic plasma cells revealed that while *Irf4* is required for the initial upregulation of *Prdm1* during differentiation, the sustained expression of *Prdm1* appears to be *Irf4* independent. These *Irf4* deficient plasma cells had altered metabolism and an increase in mitochondrial mass, which suggests that *Irf4* may play a role in the regulation of ASC metabolism (Low et al., 2019).

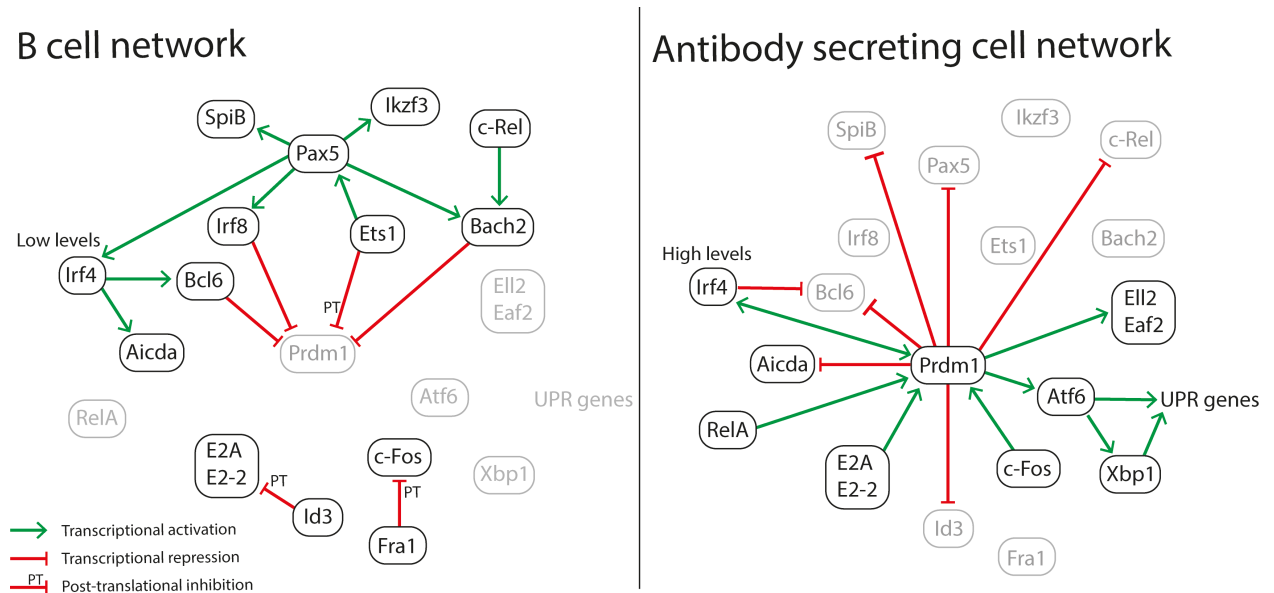


Figure 1.2 The genetic networks that regulate B cell differentiation

This figure depicts the interactions between the key genes that positively and negatively regulate B cell differentiation. Interaction networks are shown for B cells (left) and antibody secreting cells (right). Gene names are shown within the shapes. Genes that are expressed are shown in black. Genes that are not expressed or are only expressed at low levels are shown in grey. The lines indicate the direct transcriptional regulation of the protein encoded by that gene to the linked target gene. Arrowheads indicate the direction of the regulation. Lines labelled with PT indicate post-translational inhibition of the protein encoded by the linked target gene.

1.5.2. B lymphocyte induced maturation protein 1

B lymphocyte induced maturation protein 1 (Blimp-1), encoded by *Prdm1*, was first identified as a positive regulator of B cell differentiation in the BCL1 cell line (Turner et al., 1994). Ectopic expression of Blimp-1 induces both BCL1 cells and primary mouse B cells to secrete IgM, suggesting that expression of Blimp-1 is sufficient to promote differentiation (Piskurich et al., 2000; Schliephake and Schimpl, 1996; Turner et al., 1994). Blimp-1 is not required for the initiation of the B cell differentiation process, as *Prdm1*^{-/-} B cells can form pre-plasmablasts (Kallies et al., 2007). It is, however, essential for the generation of ASCs, both *in vitro* and *in vivo* (Shapiro-Shelef et al., 2003). During differentiation, Blimp-1 represses the expression of key B cell genes including *c-myc*, *Pax5*, *Bcl6*, *Spib* and *Ciita* (Lin et al., 2002; Lin et al., 1997; Minnich et al., 2016; Piskurich et al., 2000; Shaffer et al., 2002). Blimp-1 repression of *Pax5* is thought to be a key early step in the differentiation process as *Pax5* is a negative regulator of B cell differentiation. However, Blimp-1 is not the only factor required to repress *Pax5*, as downregulation of *Pax5* occurs in *Prdm1*^{-/-} B cells (Kallies et al., 2007). Blimp-1 regulates gene expression by recruiting chromatin-modifying complexes, including the BAF, NuRD and PRC2 complexes, to its target genes (Minnich et al., 2016).

Unlike *Irf4*, *Prdm1* is dispensable for the survival of fully differentiated plasma cells as ASCs are detectable *in vivo* following tamoxifen inducible deletion of Blimp1 in *Prdm1*^{fl/-}-*CreERT2*^{T/+} mice (Tellier et al., 2016). Interestingly, the expression many ASC genes is largely unaffected by the deletion of *Prdm1* and many genes that are directly repressed by Blimp-1 during the differentiation process are not re-expressed. This suggests that while Blimp-1 is required for establishing the ASC genetic network during differentiation, it is largely dispensable for its maintenance. However, these surviving *Prdm1*^{-/-} ASCs have an altered morphology and a severe reduction in antibody secretion (Tellier et al., 2016). These defects can be explained in part by the reduced expression and activation of UPR regulators, including *Xbp1*, in *Prdm1*^{-/-} ASCs. The UPR facilitates high levels of antibody synthesis and will be discussed in detail in the following section. Additionally, Blimp-1 directly induces expression of *Eli2*, an elongation factor that promotes the production of secretory immunoglobulin

transcripts over membrane bound transcripts, and its activator *Eaf2* (Kong et al., 2005; Martincic et al., 2009; Minnich et al., 2016; Park et al., 2014; Tellier et al., 2016). Furthermore, *Prdm1*^{-/-} pre-plasmablasts have reduced chromatin accessibility at the 3' enhancers of *Igh* and *Igk*, suggesting that Blimp-1 may indirectly regulate immunoglobulin transcription (Minnich et al., 2016). Together, this points to a role for Blimp-1 directly and indirectly promoting antibody secretion.

1.5.3. X-box binding protein 1

X-box-binding protein-1 (Xbp1) is a regulator of the UPR and ubiquitously expressed, but is upregulated in protein-secreting cells, including ASCs (Iwakoshi et al., 2003). During B cell differentiation, both the expression and processing of Xbp1 into its transcriptionally active form is increased (Gass et al., 2002; van Anken et al., 2003). Xbp1 regulates many of the morphological characteristics of plasma cells. Expression of the transcriptionally active form of Xbp1 in B cell lines is sufficient to drive an expansion in cell size and increases in ER, mitochondria, ribosome and lysosome content. Conversely, knockdown of *Xbp1* expression causes a decrease in cell size (Shaffer et al., 2004). Xbp1 also increases the secretory capacity of cells by inducing expression of genes involved in ER homeostasis and secretory protein production (Shaffer et al., 2004).

Early studies using *Xbp1*^{-/-}/*Rag2*^{-/-} chimeric mice proposed that Xbp1 is essential for both ASC differentiation and antibody secretion (Reimold et al., 2001). However, subsequent work using B cell specific *Xbp1* deficient mice has demonstrated that differentiation can occur without *Xbp1* expression (Taubenheim et al., 2012; Todd et al., 2009). *Xbp1* expression is also not required for the survival of bone marrow plasma cells (Tellier et al., 2016). *Xbp1*^{-/-} ASCs express key plasma cell genes including *Prdm1*, *Irf4* and *Cd138*, however, the UPR activity and antibody secreting capacity of these cells is severely reduced (Taubenheim et al., 2012; Tellier et al., 2016; Todd et al., 2009).

1.5.4. Other known positive regulators of ASC differentiation

While Blimp-1, Xbp1 and Irf4 are considered to be the key regulators of ASC biology, several other factors have been implicated in the positive regulation of plasma cell development. In contrast to c-Rel, RelA, another subunit of the canonical NF- κ B signalling pathway, is required for the upregulation of *Prdm1* following activation and *Rela*^{-/-} B cells have reduced differentiation (Heise et al., 2014). B cells that constitutively express *c-Fos* display increased differentiation *in vitro* and upregulation of *Prdm1* at earlier timepoints than their WT counterparts (Ohkubo et al., 2005). The E-proteins E2A and E2-2 are both required for efficient differentiation as B cells lacking both proteins are unable to express *Prdm1* and therefore are unable to differentiate (Gloury et al., 2016; Wöhner et al., 2016).

1.6. Protein homeostasis in antibody secreting cells

The major function of ASCs is antibody secretion; therefore, they perform a massive amount of protein synthesis, post-translational modifications and exporting proteins from the cell. Approximately 70 % of gene transcription in plasma cells is devoted to immunoglobulin expression and all of the resulting immunoglobulin must be correctly folded and post-translationally modified within the ER (Shi et al., 2015). This makes ASCs particularly sensitive to ER stress and, as a consequence, they are dependent on ER stress responses including the UPR and the ER associated degradation pathway (ERAD). The ERAD pathway ensures that unfolded or misfolded proteins are removed from the ER lumen and marked with ubiquitin for subsequent proteasomal degradation (Shenkman and Lederkremer, 2019; Sun and Brodsky, 2019). The UPR is a conserved pathway which allows cells to handle ER stress that is caused by the accumulation of unfolded or misfolded proteins within the ER lumen (Grootjans et al., 2016; Karagöz et al., 2019).

1.6.1. The ER-associated degradation pathway

Following their translation by ER-associated ribosomes and translocation into the ER lumen, newly synthesized polypeptides are bound by chaperones, including Grp78/*Hspa5* (also known as BiP) which facilitate correct protein folding. At this

stage, proteins will also undergo post-translation modifications including disulfide bond formation by oxidoreductases and *N*-linked glycosylation by glycosyltransferases. These post-translational modifications guide correct protein folding and dictate whether unfolded proteins are retained or expelled from the ER (Braakman and Hebert, 2013). The addition of complex *N*-linked glycans onto these nascent polypeptides is particularly important for the regulation of misfolded protein removal through the ERAD pathway (Smith et al., 2011; Sun and Brodsky, 2019). The glycans present on unfolded proteins are susceptible to modification by ERman1 and ER degradation enhancing alpha-mannosidase-like (EDEM) proteins. If proteins are not efficiently correctly folded, ERman1 and EDEM proteins trim glycan chains exposing a terminal alpha-1,6 linked mannose residue that serves as the signal for removal from the ER (Hosokawa et al., 2001; Ninagawa et al., 2014; Shenkman et al., 2018). Os9 and Xtp-3B/*Erlec1* bind to these trimmed glycans and deliver them to Sel1l and the E3 ubiquitin ligase Hrd1 (Christianson et al., 2008; Groisman et al., 2011; Hosokawa et al., 2009; Yamaguchi et al., 2009). Misfolded proteins are then polyubiquitinated and pulled from the ER into the cytosol by p97/*Vcp* (Jarosch et al., 2002; Rabinovich et al., 2002; Ye et al., 2001). Once in the cytosol, these polyubiquitinated misfolded proteins are degraded by the cytosolic-proteasome system (Preston and Brodsky, 2017).

1.6.2. The mammalian unfolded protein response

When the ERAD pathway is insufficient to limit the accumulation of misfolded proteins within the ER, the UPR can become activated. This pathway functions to alleviate ER stress and restore protein homeostasis, however, if this is not possible it will activate cell death pathways (Hetz and Papa, 2018). In addition to its function as a chaperone protein, Grp78 also binds to the ER stress sensor proteins Perk, Ire1a and Atf6a, and keeps them in an inactive state (Wang et al., 2017). When the protein folding capacity of the ER is reached and unfolded proteins accumulate within the ER lumen, Grp78 dissociates from ER stress sensors to bind to unfolded proteins, leaving the stress sensors free to activate the UPR (Bertolotti et al., 2000; Kozutsumi et al., 1988; Pincus et al., 2010). There is increasing evidence that the ER stress sensors may also be directly activated

by unfolded proteins (Karagöz et al., 2019). The direct binding of unfolded proteins by Ire1a induces a conformational change to an active state which allows oligomerisation (Karagöz et al., 2017). Perk has also been demonstrated to have polypeptide binding capabilities (Wang et al., 2018). However, it is still unclear whether this direct activation can occur while the ER stress sensors are still bound by Grp78.

Once released from Grp78, Atf6a transits to the golgi apparatus where it is cleaved by golgi proteases S1P and S2P, releasing the p50 fragment (Shen et al., 2002; Ye et al., 2000). This p50 fragment then translocates to the nucleus where it can drive the transcription of UPR and ERAD genes including *Xbp1*, *Hspa5*, *Hrd1* and EDEM genes (Haze et al., 1999; Yamamoto et al., 2007; Yoshida et al., 2003; Yoshida et al., 2001). Following Grp78 dissociation, Ire1a dimerises, its endoribonuclease activity is activated, and it splices *Xbp1* mRNA (Calton et al., 2002; Korennykh et al., 2009; Yoshida et al., 2001). This unconventional splicing event generates a translational frameshift in *Xbp1* which encodes the transcriptionally active form sXbp1. This active sXbp1 can then transit to the nucleus and drive expression of ERAD and additional UPR genes (Lee et al., 2003; Yoshida et al., 2001). Chronic ER stress will result in the oligomerisation of Ire1a which can lead to cell death (Ghosh et al., 2014). After release from Grp78, Perk dimerises and autophosphorylates (Bertolotti et al., 2000; Ma et al., 2002). This activated Perk will then phosphorylate eIF2a, resulting in a global inhibition of protein translation to reduce ER stress (Harding et al., 2000; Harding et al., 1999). This cellular state favours the translation of *Atf4* mRNA (Harding et al., 2000). Atf4 will drive expression of Gadd34, which dephosphorylates eIF2a, restoring translation (Novoa et al., 2001). Atf4 can also induce the expression of the pro-apoptotic Chop (Harding et al., 2000). Prolonged Perk activation and Chop expression will result in cells undergoing apoptosis (Hetz and Papa, 2018).

1.6.3. The unfolded protein response in antibody secreting cells

ASCs perform a massive amount of antibody synthesis which means that are constantly inducing ER stress. Consequentially, ASCs require their UPR to be constitutively active without activating terminal cell death pathways. To address this, ASCs have an adapted UPR pathway that allows them to accommodate sustained high levels of protein production (Grootjans et al., 2016). The Ire1a and Atf6 arms of the UPR are both active during B cell differentiation, as phosphorylated Ire1a, cleaved Atf6 and sXbp1 can be detected following differentiation (Calton et al., 2002; Gass et al., 2002; Iwakoshi et al., 2003; Zhang et al., 2005). As discussed in the previous section, sXbp1 is a key regulator of plasma cell biology and is essential for antibody secretion. The Perk/Atf4/Chop arm of the UPR, however, is selectively inactivated in ASCs. Perk activation, eIF2a phosphorylation and expression of downstream genes Chop and Gadd34 does not occur during differentiation, and is only detectable following treatment with pharmacological ER stress inducing agents (Gass et al., 2002; Gass et al., 2008; Ma et al., 2010; Zhang et al., 2005). This arm of the UPR appears to be completely dispensable for ASC biology as differentiation, survival and antibody secretion is not affected in *Perk*^{-/-} B cells (Gass et al., 2008).

It was initially proposed that activation of the UPR during differentiation was the result of increased Ig synthesis causing an accumulation of unfolded proteins in the ER (Iwakoshi et al., 2003). However, increased expression of UPR components occurs prior to cells increasing their antibody production (Gass et al., 2002; van Anken et al., 2003). Furthermore, the upregulation and splicing of *Xbp1* occurs normally in ASCs that are unable to increase immunoglobulin synthesis or produce secreted antibody (Hu et al., 2009). Interestingly, Blimp-1 appears to directly regulate the expression of *Ern1*, which encodes Ire1a, and *Atf6* in ASCs, suggesting that Blimp-1 may play a central role in this specialised plasma cell UPR (Tellier et al., 2016). Together, this suggests that the upregulation of *Xbp1* in ASCs is part of the differentiation process, not a consequence of increased immunoglobulin synthesis.

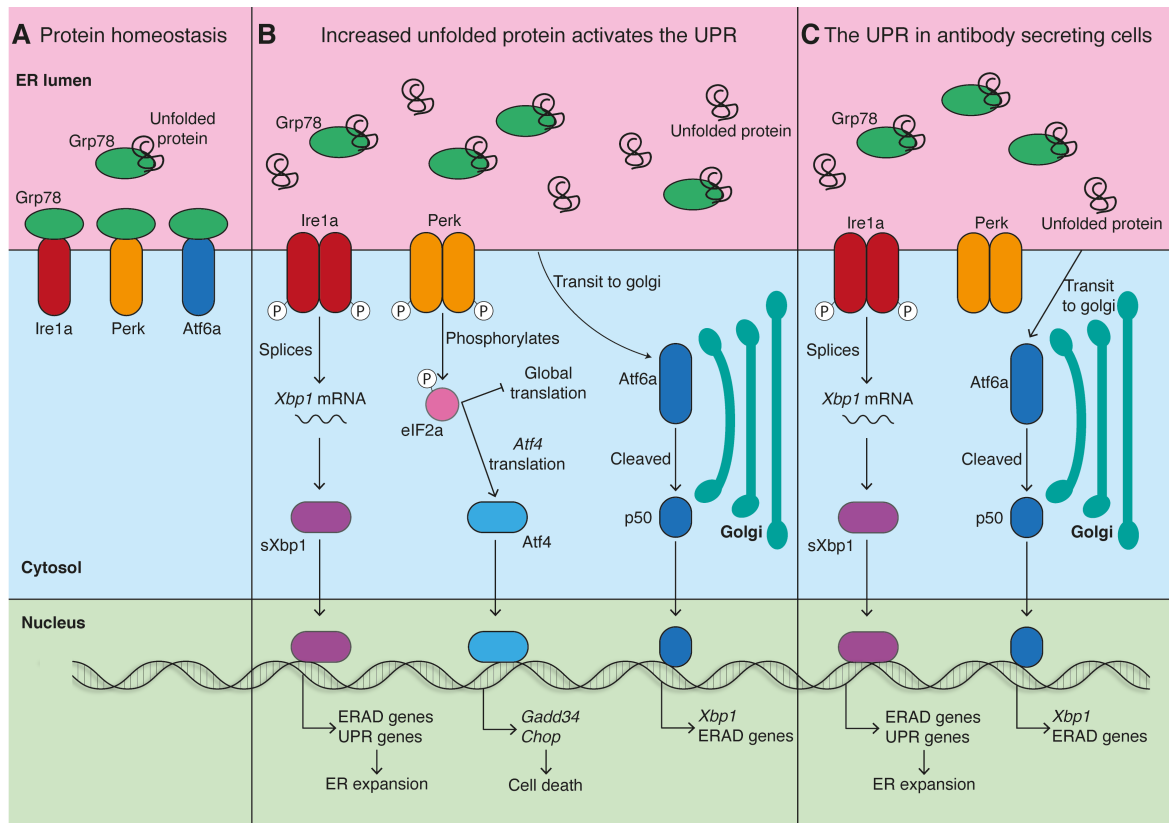


Figure 1.3 Activation of the unfolded protein response

(A) When the endoplasmic reticulum-associated degradation (ERAD) pathway is sufficient to remove misfolded proteins from the ER and maintain protein homeostasis, the protein chaperone Grp78 is bound to both unfolded proteins and the ER stress sensors Ire1a, Perk and Atf6a. (B) When the concentration of unfolded proteins increases in the ER lumen, Grp78 dissociates from the ER stress sensors, leaving them free to activate the UPR signalling pathway. Ire1a splices *Xbp1* mRNA, allowing the translation of the transcriptionally active form sXbp1. sXbp1 can transit to the nucleus and activate expression of ERAD and UPR genes, leading to ER expansion. Perk phosphorylates eIF2α, causing a block in global translation. This cellular state favours the translation of Atf4. Atf4 induces transcription of *Gadd34* and the pro-apoptotic *Chop*. Prolonged *Chop* expression leads to cell death. Atf6a transits to the golgi where it is cleaved, releasing the p50 fragment. p50 then drives expression of *Xbp1* and ERAD genes. (C) Antibody secreting cells have a modified UPR. The Ire1a and Atf6a arms of the UPR are active in ASCs, but the Perk/Atf4/Chop pathway is selectively deactivated.

1.7. The antibody secreting cell gene signature

ASCs are a group of highly specialised, terminally differentiated cells and despite differences in location, proliferation status or life-span, bone marrow plasma cells, splenic plasma cells and plasmablasts share a core transcriptional signature (Shi et al., 2015). This signature was defined by comparing the transcriptomes of each ASC subset with the transcriptional profiles of mature B cell subsets and GC B cells. This work defined the ASC gene signature as 301 genes which were upregulated in ASC compared to non-ASCs (≥ 3 -fold increase in expression in ASC subsets compared to other populations, False Discovery Rate (FDR) ≤ 0.05 , ≥ 32 fragments per kilobase of exon length per million mapped fragments (FPKM) in each ASC subset). Known regulators of ASC function including *Irf4*, *Prdm1* and *Xbp1* are in the ASC gene signature, however, most of the genes within the signature have not been previously implicated in ASC biology and some genes do not have a known function. It is highly likely that interrogation of this gene signature will reveal a role for some of these genes in the generation and functioning of ASCs.

1.8. CRISPR/Cas9 genome editing

Much of the work presented in this thesis uses clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing technology. Originally discovered as a bacterial defence mechanism, this system has been adapted for genome manipulation in mammalian cells (Cong et al., 2013; Mali et al., 2013; Wiedenheft et al., 2012). One area where this technique has been particularly useful is in high-throughput screens to examine gene function. In *Streptococcus pyogenes*, two RNAs, a CRISPR-RNA (crRNA) and a trans-activating CRISPR-RNA (tracrRNA), form a duplex which directs Cas9 to a DNA sequence that is complementary to the crRNA sequence, provided that there is the motif NGG adjacent to the complementary sequence (Deltcheva et al., 2011; Mojica et al., 2009). Once the target sequence is recognized, Cas9 will cleave the target DNA, creating a double stranded DNA (dsDNA) break (Garneau et al., 2010). For use as a programmable gene-editing system, chimeric crRNA-tracrRNA constructs (gRNAs) have been engineered and Cas9 endonuclease from *Streptococcus pyogenes* has been modified for expression in mammalian cells. Following the

introduction of a dsDNA break in the genome, DNA repair mechanisms are activated, in particular the non-homologous end-joining (NHEJ) pathway. NHEJ is a highly error-prone system of DNA repair and frequently results in the random addition or deletion of nucleotides at the break site (Gallagher and Haber, 2018). This process creates a simple yet efficient and precise mechanism of gene editing in mammalian cells (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013). This system can theoretically target any genomic region of interest, providing there is an NGG motif present.

1.9. Thesis aims

Combining transcriptomics studies with CRISPR/Cas9 gene editing presents a powerful way to uncover novel regulators of a range of cellular processes. This study aims to reveal novel genes that influence the process of B cell terminal differentiation and of ASC function. These results will provide a more comprehensive understanding of the complex genetic network that orchestrates ASC biology. It is likely that some of the genes identified within this study will be novel targets for manipulating the humoral immune response through therapeutic treatment. Furthermore, this work may highlight new avenues to specifically target the ASC population, which would be an invaluable tool in the future treatment of both antibody-mediated autoimmune conditions and ASC malignancies.

I hypothesise that the genes which are differentially regulated between mature B cells and ASC likely contain novel regulators of ASC differentiation and function. I propose that performing CRISPR/Cas9-mediated genetic perturbation screens on these differentially regulated genes will reveal novel regulators of B cell differentiation, proliferation, survival and antibody secretion.

The major goal of this thesis is to design and perform CRISPR/Cas9 screens in primary mouse B cells to identify novel regulators of B cell biology. This goal is addressed by two aims:

Aim 1: Design and perform an arrayed CRISPR/Cas9 screen on primary mouse B cells to identify positive regulators of ASC differentiation, survival, proliferation and antibody secretion

Aim 2: Design and perform a CRISPR/Cas9 screen on primary mouse B cells to identify negative regulators of ASC differentiation

This thesis also addresses a previously undescribed role of the key B cell and ASC regulator *Irf4* within the B1 cell population.

Aim 3: Investigate the role of the transcription factor *Irf4* in B1 cell biology

Chapter 2. Materials and methods

2.1. Materials

Table 2.1 Buffer components

Reagent	Supplier	Catalogue number
Dulbecco's phosphate-buffered saline (DPBS)	Gibco	14190-144
Roswell Park Memorial Institute medium (RPMI) 1640 with penicillin and streptomycin	Produced in house	-
Dulbecco's modified eagle medium (DMEM)	Gibco	11885-084
Foetal calf serum (FCS)	Gibco	10270-106
HEPES buffer solution (1M)	Gibco	15630-080
Sodium pyruvate	Gibco	11360-070
L-Glutamine (200mM)	Gibco	25030-081
MEM Non-essential amino acid solution (NEAA)	Sigma-Aldrich	M7145
β -mercaptoethanol	Sigma-Aldrich	M7522
Bovine serum albumin	Sigma	A3059
<i>N,N</i> -Dimethylformamide (DMF)	Sigma-Aldrich	319937
Tween-20	Sigma	P1379
3-amino-9-ethylcarbazole (AEC)	Sigma	A5754
Hydrogen peroxide solution	UNIVAR	2014
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)	Sigma	A1888
Citric acid	Sigma-Aldrich	251275
Trisodium citrate dihydrate	Sigma-Aldrich	S1804
Sodium acetate	BDH/AnalaR	10236.4Q
Sodium carbonate	Sigma-Aldrich	S7795
Sodium bicarbonate	Sigma-Aldrich	S5761

Table 2.2 Buffer and media recipes

Buffer/Media	Recipe
B cell media	RPMI 1640, 10 % FCS, 2 mM L-Glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 μ M β -mercaptoethanol, 1 % NEAA
293T culture media	DMEM, 10 % heat inactivated FCS, 100 μ g/mL streptomycin, 100 U/mL penicillin
Magnetic activated cell sorting (MACS) buffer	DPBS, 2 mM EDTA, 0.5 % BSA
Fluorescence-activated cell sorting (FACS) staining buffer	DPBS, 2 % FCS
Enzyme-linked immunosorbent assay (ELISA)/Enzyme-linked Immunospot (ELISpot) block solution	DPBS, 1 % FCS, 0.002 % Tween-20 and 0.6 % skim milk powder
ELISA/ELISpot wash buffer	DPBS, 0.04 % Tween-20
ABTS substrate solution	10.5 mg/mL citric acid, 15 mg/mL trisodium citrate dihydrate, 0.03 % hydrogen peroxide, 0.54 mg/mL ABTS
ELISpot substrate solution	98 mL 0.05 M sodium acetate (pH 5.0), 2 mL DMF, 25 mg AEC, with 0.03 % hydrogen peroxide added directly before use
Carbonate buffer	6.36 mg/mL sodium carbonate, 11.6 mg/mL sodium bicarbonate

Table 2.3 Tissue culture reagents

Reagent	Supplier	Catalogue number
LPS derived from Salmonella Typhosa	Sigma-Aldrich	L7895
Recombinant murine Interleukin (IL)-4	R&D	404-ML
Recombinant murine IL-5	R&D	405-ML
Recombinant murine CD40L	R&D	8230-CL
Mouse naïve B cell isolation kit	Miltenyi Biotech	130-090-862
Cell Trace Yellow (CTY)	Invitrogen	C34573A
EDTA tetrasodium salt dihydrate (EDTA)	Sigma-Aldrich	ED4SS
Percoll	GE Healthcare	17089101
Trypsin-EDTA solution	Sigma-Aldrich	59430C

Table 2.4 Antibodies for flow cytometry

Antibody	Clone	Supplier	Catalogue number
BV650 anti-mouse CD138	281-2	BioLegend	142518
PE anti-mouse CD138	281-2	BD Biosciences	553714
PE anti-mouse CD5	53-7.1	BD Biosciences	553022
PB anti-mouse CD45.1	A20	eBioscience	48-0453-82
FITC anti-mouse CD45.2	S.450-15-2	Produced in house	-
APC anti-mouse B220	RA3-6B2	eBioScience	17-0452-83
PB anti-mouse B220	RA3-6B2	BioLegend	103227
PE anti-mouse B220	RA3-6B2	eBioScience	12-0452
PE-Cy7 anti-mouse CD23	B3B4	BioLegend	25-0242

APC-eFluoro-780 anti-mouse IgD	11-26c.2a	eBioScience	47-5993-80
PB anti-mouse CD19	ID3	Produced in house	-
PE-Cy7 anti-mouse CD19	ID3	eBioScience	25-0193-81
APC-eFluoro-780 anti-mouse CD19	ID3	eBioScience	47-0193-82
APC anti-mouse CD93	REA298	Miltenyi Biotech	130-104-158
PB anti-mouse CD4	Gk1.5	eBioScience	48-0041-82
PB anti-mouse Cd11b	M1/70	eBioScience	48-0112-82
Biotin anti-mouse TCRb	H57-597	BD Biosciences	553169
Biotin anti-mouse Gr-1	RB6-8C4	eBioScience	13-5931-85
Biotin anti-mouse CD8	53.6.7	Produced in house	-
Biotin anti-mouse Ter119	TER119	Produced in house	-
Biotin anti-mouse IgM	331.12	Produced in house	-
PerCP-Cy5.5 anti-mouse IgM	331.12	eBioScience	46-5780-82
PE anti-mouse IgM	331.12	Produced in house	-
APC anti-mouse CD98	RL388	BioLegend	128210
APC anti-mouse CD21	7E9	BD Biosciences	561770

Table 2.5 ELISA and ELISpot reagents

Reagent	Supplier	Catalogue number
Polyclonal goat anti-mouse IgM	Southern Biotech	1020-01
Polyclonal goat anti-mouse IgM-HRP	Southern Biotech	1020-05
Mouse IgM kappa standard (TEPC183)	Sigma-Aldrich	M3795
96-well Multiscreen HA plates	Millipore	MAHAS4510

Table 2.6 Flow cytometry reagents

Reagent	Supplier	Catalogue number
BD Cytofix/Cytoperm	BD Biosciences	554714
SYTOX Blue dead cell stain	ThermoFisher Scientific	S34857
Fluorogold	Sigma-Aldrich	39286
Fixable viability dye (FVD) eFluoro-780	eBioscience	65-0865-14
Propidium iodide (PI)	Sigma-Aldrich	P4864
FVD eFluor506	ThermoFisher Scientific	65-0866-14
Streptavidin-PB	BioLegend	405225
Sphero rainbow calibration particles	BD Biosciences	556288
2.4G2	Produced in house	-

Table 2.7 Nucleic acid isolation reagents

Reagent	Supplier	Catalogue number
RNeasy plus Minikit	Qiagen	74134
RNeasy plus Microkit	Qiagen	74034
QIAprep Spin Minikit	Qiagen	27106
QIAprep Spin Maxikit	Qiagen	12163
DNeasy blood and tissue	Qiagen	69504
QIAshredder	Qiagen	79654
LB Broth	Produced in house	-
Carbenicillin	Sigma-Aldrich	C1613

Table 2.8 Lentiviral reagents

Reagent	Supplier	Catalogue number
Fugene6 transfection reagent	Promega	E2692
Polybrene	Sigma-Aldrich	H9268
RetroNectin	Produced in house	-

Table 2.9 CRISPR gRNA sequences

gRNA name	Strand	Targeted exon	Sigma ID	gRNA sequence
1700017B05Rik-1	1	2	MM5000027251	CATACATCATAGGCCCCAGAGG
1700017B05Rik-2	1	2	MM5000027252	CCTGGCTGCTAGCAGAAAGGGG
1700021K19Rik-1	1	3	MM5000012045	CTGCCAGTAATCAGCCTGGCGG
1700021K19Rik-2	-1	3	MM5000012046	TTGCCGCCAGGCTGATTACTGG
4632428N05Rik-1	1	3	MM5000004703	TGCATGGCGTCTAATGAGCAGG
4632428N05Rik-2	-1	3	MM5000004704	TCATTAGACGCCATGCATGTGG
A430078G23Rik-1	-1	3	MM5000027273	AGGTACTCGCTGCCCTGAAGGG
A430078G23Rik-2	-1	3	MM5000027274	TCTCCAGCTGCGTCTTTGTGG
AB124611-1	-1	2	MM5000005379	ACATCAGGCCGATCTGAGGGGG
AB124611-2	-1	2	MM5000005380	GATGTAGATGGGGTCCTCGTGG
Abcb1b-1	-1	3	MM5000035463	TCCCAAATACGCCAACAGCAGG
Abcb1b-2	1	3	MM5000035464	AACCTGCTGTTGGCGTATTTGG
Ada-1	-1	2	MM5000013985	AGTTACACGTCCACCTGGATGG
Ada-2	-1	2	MM5000013986	CAGAAACCATCTTATACTTTGG
Adamts6-1	1	3	MM5000026499	GACCTTCTTGATCCACAGCAGG
Adamts6-2	-1	3	MM5000026500	CATTTTGGTCAACTCTTATTGG
Adk-1	1	4	MM5000021559	ACGCAGAATTCAATGAAAGTGG
Adk-2	1	5	MM5000021560	CTTGTGCTGCGTGTATCACTGG
Aff3-1	-1	3	MM5000035347	GGACAGGAATGCGTTACGGAGG
Aff3-2	1	3	MM5000035348	TCCGTAACGCATTCTGTCCGG
Akt3-1	-1	3	MM5000034041	GGCTCATTCATAGGCTATAAGG
Akt3-2	1	4	MM5000034042	AATGTTCTCTCTATAACAGTGG
Alad-1	1	5	MM5000025135	CGGACAGCCTCAATAGTTGGGG
Alad-2	1	5	MM5000025136	GACGGACAGCCTCAATAGTTGG
Alcam-1	1	2	MM5000034047	GAGGTACATCGAGACGGCAAGG
Alcam-2	1	2	MM5000034048	AGACGGCAAGGCATGACAATGG
Aldh18a1-1	1	4	MM5000015989	CTGAGGGGTACCGTGATAAAGG
Aldh18a1-2	1	4	MM5000015990	GAGGGGTACCGTGATAAAGGGG
Aldh9a1-1	-1	2	MM5000000115	TCTCCTGAACACGCGAAAGTGG
Aldh9a1-2	-1	2	MM5000000116	GAGGACTTGGCAGCGCTCCAGG
Alg2-1	-1	2	MM5000025159	CTCCGAGCAACGAGCACTTTGG
Alg2-2	1	2	MM5000025160	TGCCAAAGTGCTCGTTGCTCGG
Ap2m1-1	1	6	MM5000021331	AGATTGGCTGGCGGCGAGAAGG
Ap2m1-2	1	3	MM5000021332	GTGCTCATATACGAGCTGCTGG
Apbb1ip-1	1	4	MM5000014505	GATGCCCTCATGGCAGATCTGG
Apbb1ip-2	1	4	MM5000014506	GCCGAACAGAGAACCATCCAGG
Arf4-1	1	3	MM5000017427	AGATAAAATTAGGCTCTCTGG
Arf4-2	1	5	MM5000017428	CAGGATCTGCCAAACGCTATGG
Arfgap3-1	-1	2	MM5000006587	TGGGCCAGCATAAGCTATGGGG

Arfgap3-2	1	2	MM5000006588	CAAGTGAACCCCGAGCGACCGG
Arhgap26-1	1	3	MM5000016191	CAGGAATCTTGAAGACGAGCGG
Arhgap26-2	1	4	MM5000016192	GAGGTGCTCATCACACCCTTGG
Arhgap4-1	-1	2	MM5000033701	AACGCTTTACCAGCCGCACTGG
Arhgap4-2	1	2	MM5000033702	GCGTTCAGCCAACTTGTCCAGG
Arhgef18-1	1	4	MM5000004991	TGAGTTTGAGGCCGAGTCCTGG
Arhgef18-2	-1	4	MM5000004992	ACAGAGAGGCTCCAGGACTCGG
Arhgef3-1	-1	3	MM5000021021	TCTGGCTGAAGCGCTTTAATGG
Arhgef3-2	-1	3	MM5000021022	CTGAAGCGCTTTAATGGTGTGG
Asns-1	-1	3	MM5000025189	ACCATCTGTATGCTGGACGGGG
Asns-2	-1	3	MM5000025190	CAGAGACACCTATGGAGTCAGG
Atat1-1	1	3	MM5000006963	CTTTCATCCTCAAAGCGCTGG
Atat1-2	1	3	MM5000006964	CAGTATGTAAATAACGTGCCGG
Atf5-1	-1	3	MM5000007279	GCTGTTTACTGCCGCACTGAGG
Atf5-2	-1	3	MM5000007280	CGGGAGATCCAGTACGTGAAGG
Atl2-1	-1	7	MM5000038311	TATGAGCACTCATATGGCTTGG
Atl2-2	1	7	MM5000038312	AGCCATATGAGTGCTCATAAGG
Atp6v0a1-1	-1	4	MM5000038135	CGATCATGTCCCGAGGGAAGGG
Atp6v0a1-2	-1	4	MM5000038136	TTCACCGGTGTCCATAATTGGG
B3gnt8-1	1	2	MM5000023501	TCAGCACTGCTCACACTCCTGG
B3gnt8-2	1	2	MM5000023502	GTTGCTGCCTCGGAGATCTTGG
Bach2-1	1	6	MM5000008871	GGAGTTCATCCACGACATCCGG
Bach2-2	-1	6	MM5000008872	TTCTCTTTTCGACAGCGCTGGG
Bank1-1	1	2	MM5000008821	CTGACACTTCTTTGGCGTCAGG
Bank1-2	1	2	MM5000008822	TGACAGAGATGTAACCATCCGG
BC003331-1	-1	3	MM5000000481	AAACAGCCGAGAGCTAAGTTGG
BC003331-2	-1	3	MM5000000482	AGAGCTAAGTTGGATAATTTGG
BC094916-1	1	3	MM5000035693	GTGTACTCCTCCCTGATTCTGG
BC094916-2	-1	3	MM5000035694	TGCAGAACCAGAATCAGGGAGG
Bckdk-1	1	3	MM5000035235	CTCTATTCCGGTCGCTCACAGG
Bckdk-2	-1	3	MM5000035236	CTGCCATCCTGTGAGCGACCGG
Bcl11a-1	1	3	MM5000004435	AGTGCAGAATATGCCCCGCAGG
Bcl11a-2	1	3	MM5000004436	CCTCTCCTCGGTCTGCACACGG
Bcl6-1	-1	4	MM5000038233	GCATGTTGTGACACATGCAGG
Bcl6-2	1	4	MM5000038234	ATCTGCAGGTACATGGCTGTGG
Bcl9l-1	1	5	MM5000005443	AATGTAGCAGACCCAGCCATGG
Bcl9l-2	1	5	MM5000005444	TCAGTGAGAGCAGTGCACCAGG
Bet1-1	1	2	MM5000009289	CAAGCATTATAACCAGTATTGG
Bet1-2	-1	2	MM5000009290	ACTATGGCTATGCCAATACTGG
Bhlha15-1	-1	2	MM5000033769	CTGTGTAGAGTAGCGTTGCAGG
Bhlha15-2	1	2	MM5000033770	AAGATCGAGACCCTCACGCTGG
Bin1-1	1	4	MM5000040755	GGAGGTGTATGAGCCCGAGTGG

Bin1-2	1	3	MM5000040756	AAGGATCTTCGGACCTATCTGG
Bst2-1	1	4	MM5000034985	TGACACTTTGAGCACCAGTAGG
Bst2-2	-1	3	MM5000034986	GAAGTCACGAAGCTGAACCAGG
Btd-1	-1	2	MM5000011311	CACGTAGTACTCAGCCTCGCGG
Btd-2	1	2	MM5000011312	GCTGTGTACGAGCATCCATCGG
Btg1-1	-1	2	MM5000035273	CTGCTCAGTCCAATCCGCTGGG
Btg1-2	1	2	MM5000035274	TTCCCAGAAAAGCCGTGCAAGG
Cacna1h-1	1	4	MM5000015995	GTCACCCAGGTAGCATTCTCTGG
Cacna1h-2	-1	4	MM5000015996	GTGGAGATGGTTATCAAGATGG
Cacna1i-1	-1	3	MM5000026365	GGCACTCCATATCGTCACATGG
Cacna1i-2	-1	3	MM5000026366	CTCCATATCGTCACATGGCTGG
Calr-1	1	2	MM5000003025	TTGGATTTCGACCCAGCGGTTGG
Calr-2	-1	2	MM5000003026	GGCAAATTTTACGGGGACCTGG
Capn5-1	1	3	MM5000003691	TGCCATCTACGAAGAGCCGGGG
Capn5-2	1	3	MM5000003692	GATGCCATCTACGAAGAGCCGG
Cars-1	1	3	MM5000033549	CCGGGTGAGGCTATTATAGAGG
Cars-2	-1	3	MM5000033550	CCTCTATAATAGCCTCACCCGG
Ccdc117-1	-1	2	MM5000033587	AGAAAGAAACACAAGCGGTTGG
Ccdc117-2	-1	3	MM5000033588	ATGCCCAGCATGCTAGATGTGG
Ccpg1-1	1	3	MM5000013813	CCGTTCTTTATGTAGGGCATGG
Ccpg1-2	-1	3	MM5000013814	AGCTCCTCTTGTTCTACTAAGG
Cd19-1	1	2	MM5000017761	CTCCAGGAAGGGTGTTGACTGG
Cd19-2	1	2	MM5000017762	CAGGTAGAAGCCCCCATATGG
Cd2-1	-1	2	MM5000033343	TGAGGTGCGATGGGTAAGGAGG
Cd2-2	-1	2	MM5000033344	TGATGAGGTGCGATGGGTAAGG
Cd22-1	1	5	MM5000033351	GTCATTGGCACGTATCGGGTGG
Cd22-2	1	5	MM5000033352	TGTTGTCCAGACGTGCCTTGGG
Cd28-1	1	2	MM5000027817	ATGCCGAGTTCAACTGCGACGG
Cd28-2	1	2	MM5000027818	AACAGTGACGTTCCGTCTCTGG
Cd37-1	-1	2	MM5000018075	GATTTTCTGCTTCGGCACCTGG
Cd37-2	1	2	MM5000018076	GAAGCTGGTCTTGTCATGAGG
Cd40-1	-1	3	MM5000013993	CGAATCTCCCTGTTCCACTGGG
Cd40-2	-1	3	MM5000013994	TGTGCAGTGGCTTGTCAGTCGG
Cd55-1	-1	6	MM5000040639	GACTCTTATACGTATAGCCAGG
Cd55-2	-1	6	MM5000040640	TCTTATACGTATAGCCAGGTGG
Cd72-1	1	2	MM5000013915	GCTTTGTCCGCTAGTGCAGGGG
Cd72-2	-1	2	MM5000013916	TTGGCTTCCCCTGCACTAGCGG
Cd74-1	-1	2	MM5000033377	GATGGTCAGCTTGCTAGGCGG
Cd74-2	-1	2	MM5000033378	TTTCGGAAGCTTCATGCGAAGG
Cd83-1	1	2	MM5000004855	TGCAGCCTGGCACCCGCGATGG
Cd83-2	1	2	MM5000004856	GGCACCCGCGATGGCGATGCGG
Cdv3-1	-1	3	MM5000005343	ATTGGGAAGAAGGTGGAGGTGG

Cdv3-1	-1	3	MM5000005344	AAGAAGGTGGAGGTGGCAGTGG
Chid1-1	-1	3	MM5000003179	GTCCAAGATCGGGGTCTGGTGG
Chid1-2	-1	3	MM5000003180	CCTGTCCAAGATCGGGGTCTGG
Chpf-1	1	3	MM5000004671	TGTGCACAGGGTCACGCACGGG
Chpf-2	1	3	MM5000004672	ATGGGCTGTCAAGGCGCTGCGG
Chst1-1	-1	3	MM5000012865	GTGATAGAGCGGCTCGAACAGG
Chst1-2	-1	3	MM5000012866	GTGCCCCCTCGTATTGTTCTGG
Ciita-1	-1	18	MM5000035283	GAACCCCAAAGGGGATAGTGGG
Ciita-2	1	18	MM5000035284	GGACACCCACTATCCCCTTTGG
Cited2-1	1	2	MM5000038145	GAGCACATACACTACGGCGCGG
Cited2-2	-1	2	MM5000038146	TGTATGTGCTCGCCCATGAGGG
Clcf1-1	1	2	MM5000035115	TGCCAGCTCTTAATCGCACAGG
Clcf1-2	-1	2	MM5000035116	GTGCCACAGCACCGTGCATAGG
Clptm1l-1	-1	2	MM5000011347	GTCTTCCACGTTCAAGATCAGG
Clptm1l-2	-1	2	MM5000011348	CCAAGGCTGGACCGTGTGGTGG
Cltb-1	1	3	MM5000026501	TGAGTCAACCTGTCCGCCTGGG
Cltb-2	-1	3	MM5000026502	AGGCTAACGGGCCTGCCGATGG
Cnn2-1	1	2	MM5000015599	GGAAGCAGAACTCCGAAGCTGG
Cnn2-2	-1	2	MM5000015600	GAAGTCAGGGCCAATGGAGAGG
Cnn3-1	1	2	MM5000010525	CGAGGAAGATCTGCGCAACTGG
Cnn3-2	1	2	MM5000010526	AAGTATGACCAGCAGGCCGAGG
Cnpy2-1	1	3	MM5000000001	GAGTGGGAAATTGCCCGCGTGG
Cnpy2-2	-1	3	MM5000000002	CCATCTGGATTGATTCGGAAGG
Cnr2-1	-1	2	MM5000034997	TTGCAGGCGAAGATCACGCTGG
Cnr2-2	-1	2	MM5000034998	TCCGCAGGGCGTAAATGATAGG
Cnst-1	1	3	MM5000011737	ACGCAGAATGCAACGTCGCAGG
Cnst-2	-1	3	MM5000011738	GCTGCAGCGCTGGCGCTTTGGG
Cope-1	-1	2	MM5000022421	TCTACTTCCCGCTCAGGACTGG
Cope-2	1	2	MM5000022422	TCCTGAGCGGGAAGTAGAGAGG
Coro1a-1	-1	3	MM5000003751	GTAGACAAGAACGTGCCCTGG
Coro1a-2	1	3	MM5000003752	ACCAGGCGATGTCTAGCACAGG
Coro2b-1	-1	2	MM5000013455	TAGTCACTGAGAGCGCAGGAGG
Coro2b-2	1	2	MM5000013456	ACTTGGAAGTGCAGTACTGCGG
Cotl1-1	1	2	MM5000000883	TAATCTGCTCCCTGATCGCCGG
Cotl1-2	1	2	MM5000000884	CCCTGATCGCCGGGGACAATGG
Cpeb2-1	1	2	MM5000009973	ACCATCAGAACAGCGGTTGGGG
Cpeb2-2	1	2	MM5000009974	CCGCTGAAGAAGCCGTTCTCGG
Cpm-1	1	3	MM5000010693	AATACGTGGCGAATATGCACGG
Cpm-2	1	3	MM5000010694	ATTCCAGAATTCAAATACGTGG
Cpox-1	1	2	MM5000024713	TGAGCATTTCCGTCGTTTCATGG
Cpox-2	-1	2	MM5000024714	GAAAGATTCCCATGAACGACGG
Cr2-1	-1	2	MM5000032999	CACCTAGCTACCGCCTCATTGG

Cr2-2	1	2	MM5000033000	CTCCAATGAGGCGGTAGCTAGG
Creb3l2-1	-1	2	MM5000024483	GAGAAGAGCGAGTCAATGGAGG
Creb3l2-2	1	2	MM5000024484	TGAGAGGCGCTGGTGATGTTGG
Creg1-1	1	3	MM5000033013	ATTAAAAATAAGCCGTATCTGG
Creg1-2	-1	3	MM5000033014	GCAAAGAACCAGTTATGGCTGG
Creld2-1	1	2	MM5000033017	CGGCCAGGAAGAATTCGGTGG
Creld2-2	1	2	MM5000033018	AATTCGGTGGCGGCAACACGG
Ctse-1	1	3	MM5000020697	GGAAGAACTACGGGCCCAAGG
Ctse-2	-1	3	MM5000020698	GCCCGTAGTTTCTCCGAAGGG
D17Wsu104e-1	-1	2	MM5000032815	CATTCACCTACGCTTCCCAAGG
D17Wsu104e-2	1	2	MM5000032816	GTCCCTCCTTGGGAAGCGTAGG
Dap-1	-1	2	MM5000024549	CTTCCCATCTCCGGTATGTGGG
Dap-2	1	2	MM5000024550	CCACATACCGGAGATGGGAAGG
Ddost-1	-1	2	MM5000010137	GGGTCATCTGCGGTCTTGAAGG
Ddost-2	-1	2	MM5000010138	AACTCCCCGTACTTAATGAGGG
Dek-1	1	2	MM5000002599	GTTCTTCTCGGATGAGGGCGG
Dek-2	1	2	MM5000002600	CGGGTTCCTTCTCGGATGAGGG
Dennd5b-1	1	2	MM5000026819	GGATAGTGGGCCAGAACTTTGG
Dennd5b-2	-1	3	MM5000026820	TCTGACTACCCTCTTCGAGAGG
Derl1-1	-1	2	MM5000003983	GCCACCTTTTACTTCCCCGTGG
Derl1-2	1	2	MM5000003984	GGGCCCACGGGGAAGTAAAAGG
Derl3-1	1	2	MM5000021069	CTCTACTTCAACCCGCACCTGG
Derl3-2	-1	3	MM5000021070	TCCCAGGGGGCCCGAAGAAGAGG
Desi1-1	1	3	MM5000021079	TCCTTGTGTACAACCTATAGAGG
Desi1-2	-1	3	MM5000021080	ACCTCTATAGTTGTACACAAGG
Dhdds-1	1	3	MM5000026729	TTATGAACGCGATATGTTTGGG
Dhdds-2	1	3	MM5000026730	ACACTTCTTGGCATAGCGACGG
Dnajb11-1	-1	2	MM5000020997	CCTTTATGGAGGCACTTCGAGG
Dnajb11-2	1	2	MM5000020998	CCTCGAAGTGCCTCCATAAAGG
Dnajb9-1	-1	3	MM5000032743	GGACTGTCACTCAACGAAGAGG
Dnajb9-2	1	3	MM5000032744	TCATACTCTTCCGACTATTGG
Dnajc3-1	-1	2	MM5000011259	AAGGCGTCGGCTAGCTGTCCGG
Dnajc3-2	1	2	MM5000011260	GGAAGAAATTACTCGCAGCCGG
Dpagt1-1	-1	2	MM5000013389	CTCCTCCACGAAGCAGTTCAGG
Dpagt1-2	1	3	MM5000013390	AAGCCCTTCCGCTGGATTCTGG
Dtx1-1	1	4	MM5000035037	AGTTCTTCACGGGGAGCGCCGG
Dtx1-2	-1	6	MM5000035038	TCCTGAGGATGTGGTTCGGAGG
Dusp2-1	1	4	MM5000020399	GATTCAGAGCCACCGGGTACGG
Dusp2-2	-1	4	MM5000020400	TGAGCGAGAGATGCCAGCTTGG
Ebf1-1	-1	2	MM5000008227	TGGCCCTGTCTGTCGTAGAGGG
Ebf1-2	1	2	MM5000008228	ATTGAGAGGACGGCCTTTGTGG
Edem1-1	1	2	MM5000007617	CTGACTCTGGTTGATGCCTTGG

Edem1-2	-1	2	MM5000007618	AGTCAGAGAGTAATTCCCTAGG
Edem2-1	1	2	MM5000020485	CAGGTAAGTGTCTAGGCGTGG
Edem2-2	-1	2	MM5000020486	CACGCCTACGACAGTTACCTGG
Edem3-1	1	2	MM5000000323	AAATGTTTGATCATGCTTATGG
Edem3-2	1	3	MM5000000324	TCATGCCTTTAACCTGCCGGGG
Eef1a1-1	1	3	MM5000032661	AAGACCCAGGCGTACTTGAAGG
Eef1a1-2	1	3	MM5000032662	CTGGTCTCGAATTTCCACAGGG
Eil2-1	1	3	MM5000011277	AACTTCTACTTGTCAAACGTGG
Eil2-2	1	3	MM5000011278	GTGGGAAGAGATAATCCTCAGG
Eil3-1	-1	3	MM5000020289	GGCCAGTGATCGCTTTCCAAGG
Eil3-2	1	4	MM5000020290	GCCTAAGCGTTGGTACACAAGG
Endou-1	1	3	MM5000018241	TGGGCAGCGGTCATTACAGTGG
Endou-2	1	3	MM5000018242	GTGCTTGTCTAGGCTTCCCGG
Enpp1-1	-1	4	MM5000017823	ATGGACTTGCAACAAATTCAGG
Enpp1-2	-1	5	MM5000017824	TGTGAAAGCATCGATACCCCGG
Entpd1-1	1	2	MM5000017989	CTTGGTTTCACCTCTATCTTGG
Entpd1-2	1	3	MM5000017990	TGGGATTGTGTTGGATGCGGGG
Epcam-1	1	3	MM5000018011	AATGCCAGTGTAATTCCTATGG
Epcam-2	1	3	MM5000018012	GTCTGTGACAACCTACAAGCTGG
Ephx1-1	-1	3	MM5000018049	GAGGAATGAGTTTGACTGGAGG
Ephx1-2	1	3	MM5000018050	AATCTTGGTCTTAAAGTGTGGG
Epsti1-1	-1	2	MM5000018243	TCTGCCTTCTGCTCTCGTTTGG
Epsti1-2	-1	2	MM5000018244	CTCTCGTTTGGTGCTATCAGGG
Ergic-1	1	3	MM5000016853	TCTTCCTGTCTGGAGCTCACTGG
Ergic-2	-1	3	MM5000016854	ATGAAGAGGCAGCAGCAGATGG
Erlec1-1	-1	2	MM5000027673	CTTCCACTTGTGACAAGTGGGG
Erlec1-2	-1	2	MM5000027674	TACTTCCACTTGTGACAAGTGG
Erp44-1	1	4	MM5000008713	TGCTGATCACAATCAACTCTGG
Erp44-2	-1	4	MM5000008714	ATGTTGCATCCAATTTTGGAGG
Ets1-1	1	7	MM5000032587	AAGTGGGCCGATCCAGCTGTGG
Ets1-2	1	7	MM5000032588	AGTCCTTTATCAGCTGGACAGG
Evl-1	1	6	MM5000024819	GCACCACTCTCTCCTGTAGTGG
Evl-2	1	6	MM5000024820	GAGAGCTCTGCATCAGGACTGG
Faim3-1	-1	2	MM5000017829	GTGGAGCATATCCCAGGTTTGG
Faim3-2	-1	2	MM5000017830	AGTGGGCATTTCGATGATAATGG
Fam129c-1	1	2	MM5000027679	CTTCCTGCCCTGTTACCGTAGG
Fam129c-2	-1	2	MM5000027680	CCAGCTGCCTACGGTAACAGGG
Fam214a-1	1	5	MM5000032491	TTAGAGCCTGTCAGCACCATGG
Fam214a-2	1	5	MM5000032492	CCTATGCAGCAAGCACACATGG
Fam43a-1	1	1	MM5000005525	AAGCACAAGTTCGAGCTGCTGG
Fam43a-2	1	1	MM5000005526	AAGCTGCACATCACCAGCGAGG
Fam46c-1	-1	2	MM5000014065	ACAGAGGCGGAATTTAGCTGG

Fam46c-2	1	2	MM5000014066	GAGCACATGTAACGCTCCAGGG
Fam49b-1	-1	5	MM5000004161	GTGCAGTCGTGCCACTAGTAGG
Fam49b-2	1	5	MM5000004162	CTTGCAACTTCTCATCGGCTGG
Fam65b-1	-1	3	MM5000021295	CCTCCACTCTCTTCGGCTGAGG
Fam65b-2	1	4	MM5000021296	GAGTTTCACCAGACGGAGCTGG
Fcer1g-1	1	2	MM5000017647	TTGAGTCGACAGTAGAGTAGGG
Fcer1g-2	1	2	MM5000017648	GACAATACCATACAAAAACAGG
Fcer2a-1	1	3	MM5000040769	AGCAGCAACGCTTTCTAGGAGG
Fcer2a-2	1	3	MM5000040770	CACAGCAGCAACGCTTTCTAGG
Fcr11-1	1	4	MM5000032407	ATTACTGTGCGGCTAACGATGG
Fcr11-2	1	4	MM5000032408	GCTTGAAGACACGGCCCCCAGG
Fgd2-1	-1	2	MM5000017163	GCTGGTGTTCAAGGGCTATGGGG
Fgd2-2	1	2	MM5000017164	CCTGGATCCCATAGCCTTGAGG
Fkbp11-1	1	2	MM5000017141	ATGTGGAGCGTGTCTCCAATGG
Fkbp11-2	1	2	MM5000017142	ATTCCGTGCACGATTCAGGGGG
Fkbp2-1	1	3	MM5000012065	GTCCCAGCCCTTGATAACCTGG
Fkbp2-2	-1	4	MM5000012066	GAAGGGGAAAAGCGGAAGTTGG
Flna-1	1	3	MM5000013119	TGCCAGTCTCGACTGAAGTTGG
Flna-2	-1	3	MM5000013120	GCTGATCTTAGGCCTCATCTGG
Fndc3a-1	-1	3	MM5000011525	CTGGTACAGGTTAATCCAGGGG
Fndc3a-2	1	4	MM5000011526	TAGGAGGCACGGAACCATTTGG
Fndc3b-1	1	4	MM5000009113	CTGGAGGCACATGGATAGGAGG
Fndc3b-2	1	5	MM5000009114	GGCTGAGGGATAACTCGGTGGG
Fos-1	1	2	MM5000017123	GAATGGTGAAGACCGTGTGAGG
Fos-2	1	2	MM5000017124	TGGTGAAGACCGTGTGAGGAGG
Fut1-1	1	2	MM5000032329	TGTTGAGCGGGCTCCGTATAGG
Fut1-2	-1	2	MM5000032330	GCAGGGAGATTGCGAACACGGG
Fut8-1	-1	5	MM5000027703	AGCTGTCCCCTGGTCAATGGGG
Fut8-2	-1	5	MM5000027704	GTAGCTGTCCCCTGGTCAATGG
Galnt10-1	1	2	MM5000004433	AAGGACTGGCATAACAAGGAGG
Galnt10-2	1	2	MM5000004434	TAACAAGGAGGCTATCAGGAGG
Galnt2-1	1	3	MM5000038365	ACCAGGAGGCATACGTTGGCGG
Galnt2-2	1	3	MM5000038366	GCATACGTTGGCGGTACAATGG
Galnt6-1	1	4	MM5000017297	CAATGATCACGCTGGTGGTGGG
Galnt6-2	1	4	MM5000017298	AGTGGACCAGGCTTCGTTGTGG
Gfpt1-1	-1	2	MM5000007809	AGATTTCTCGTCTTGTTGAGG
Gfpt1-2	1	2	MM5000007810	CGAACAAGACGAGAAATCTTGG
Gimap3-1	1	2	MM5000032227	TGAGCCTGGACTCGAATGCTGG
Gimap3-2	-1	2	MM5000032228	ACGACCAGCATTGAGTCCAGG
Gimap8-1	1	6	MM5000032233	GGGCTGGAAAGAGTGCAACTGG
Gimap8-2	1	6	MM5000032234	GCAACTGGGAACACTATCCTGG
Glb1-1	-1	2	MM5000013459	CCAGTAGAAGCGGGGTATCCGG

Glb1-2	-1	2	MM5000013460	TTCCCGAGATGTATCGGAATGG
Glpr-1	1	3	MM5000032267	TCCCAGGGCGGTGAAATTTGGG
Glpr1-2	-1	3	MM5000032268	CACCCAAATTTACCCGCCCTGG
Glt8d1-1	-1	4	MM5000011561	CAGAGGCAGCAATAACCACGGG
Glt8d1-2	-1	4	MM5000011562	TTTTGATGAACGCTGTTTATGG
Gm6377-1	1	2	MM5000031793	ACTGGATTTATATCGATCTTGG
Gm6377-2	-1	2	MM5000031794	TACAAATTCCTGACTAGCTTGG
Gmfg-1	1	6	MM5000027623	GCAGGAAGTAAAAACAGGCTGG
Gmfg-2	1	5	MM5000027624	ACAAGTACGTGCATGATGACGG
Gne-1	-1	3	MM5000026749	CACCTGATCTCTATGTGCGAGG
Gne-2	1	3	MM5000026750	CAGAGCAAGGGCGTCAAATCGG
Gngt2-1	1	3	MM5000004065	GAAATCAAGGATTATGTAGAGG
Gngt2-2	1	3	MM5000004066	GGACAGACCCTCTTCTCAAAGG
Gpr155-1	-1	4	MM5000013055	CTGAAGAAGTCGGCGTTTGTGG
Gpr155-2	-1	4	MM5000013056	CAGAACCCGATAGTATTTATGG
Gpr183-1	-1	2	MM5000006131	TGCCTGTCCGTCTGGATTCTGG
Gpr183-2	-1	2	MM5000006132	ACCCACTCACCGAGAAATCTGG
Gpr55-1	1	2	MM5000031507	ATGAGCAGTTGTACGCTCTGG
Gpr55-2	1	2	MM5000031508	AAAGGTCTTCTGGGCGACCGG
Gsn-1	1	2	MM5000020411	GAGCACCCCGAATTCCTGAAGG
Gsn-2	-1	2	MM5000020412	CTCCACACGCCAGATCTGCAGG
Gvin1-1	1	2	MM5000039897	CACTTCTGTGAGCATCTCCTGG
Gvin1-2	1	2	MM5000039898	TATTGTAAGGCCTGGGCCGAGG
H13-1	-1	2	MM5000001779	AGTGTGCAGCTGGCGATGATGG
H13-2	-1	2	MM5000001780	GCGGCATCTCGACTGGTGATGG
H1f0-1	1	1	MM5000006909	GATGAGCCCCAAAAGGTCGGTGG
H1f0-2	-1	1	MM5000006910	GAGAACACCGGTGGTCACTAGG
Haao-1	-1	2	MM5000005067	TCAAATCATGTTGTTGGAGG
Haao-2	-1	2	MM5000005068	AGCTCAAATCATGTTGTTGG
Hdlbp-1	-1	4	MM5000026847	AACAAGATCCGGCCCATCAAGG
Hdlbp-2	1	4	MM5000026848	GGAAGGCATCCTTGTAGGTTGG
Herpud1-1	-1	4	MM5000022369	AGATGTTCTCCCATCCGGAGGG
Herpud1-2	1	4	MM5000022370	GAACCTTTCTCCCTCCGGATGG
Hhex-1	1	3	MM5000005049	TCAGAATCGCCGAGCTAAATGG
Hhex-2	-1	3	MM5000005050	CAGTCTTCTCCATTTAGCTCGG
Hid1-1	1	2	MM5000005085	GCAAAGACATCCTGCACCGAGG
Hid1-2	1	2	MM5000005086	TCCCAGAAAGCATTGTCGGTGG
Hist1h1c-1	-1	1	MM5000031235	GGTGACCTTCGCCTTCTTTGGG
Hist1h1c-2	-1	1	MM5000031236	GGCTTCTTCGCCTTCTTCGGGG
Hsd11b1-1	-1	3	MM5000006709	CCTCTGCTCACTACATTGCTGG
Hsd11b1-2	-1	3	MM5000006710	CACTACATTGCTGGCACTATGG
Hsp90b1-1	1	5	MM5000027009	CGTGTCTGTGACATGCAGCAGG

Hsp90b1-2	-1	5	MM5000027010	ATGTCACAGACACGGGTGTAGG
Hspa13-1	1	2	MM5000024527	ACACCAACCGAACAGTAGGTGG
Hspa13-2	-1	2	MM5000024528	GTACCACCTACTGTTTCGGTTGG
Hspa5-1	-1	2	MM5000025689	CACATACGACGGCGTGATGCGG
Hspa5-2	-1	2	MM5000025690	ATCGAAGACCGTGTTCTCGGGG
Htatip2-1	-1	4	MM5000023541	CTGGACTTATCGGCCCCCCTGG
Htatip2-2	-1	4	MM5000023542	GACTTATCGGCCCCCCTGGAGG
Hyou1-1	1	4	MM5000013371	TTTTAGGTGATAGTGCAGCCGG
Hyou1-2	-1	5	MM5000013372	GGGAAACGGGACCGGTAAAGGG
Icosl-1	-1	2	MM5000006537	AAAGAACCCGCTAGAAACATGG
Icosl-2	1	2	MM5000006538	TTTCTAGCGGGTCTTTTCTGG
Idh2-1	-1	2	MM5000007309	GAGAAGCCGGTAGTGGAGATGG
Idh2-2	-1	2	MM5000007310	AAGGTGGAGAAGCCGGTAGTGG
Ift20-1	-1	5	MM5000014605	AGCAAGTTCCGAGCACCAATGG
Ift20-2	-1	5	MM5000014606	GCTTCTCTCTGCTTCGCTATGG
Il4ra-1	-1	4	MM5000025613	TCCAGGAACCACTCACACGTGG
Il4ra-2	1	4	MM5000025614	TGGTTCCTGGATAGCGCTGTGG
Iqcb1-1	1	4	MM5000024953	TTCTCGAATCCAGGGTGGTTGG
Iqcb1-2	1	4	MM5000024954	CAAGATTCTTCTCGAATCCAGG
Irf4-1	1	3	MM5000006643	GTGTACAGGATTGTTCCAGAGG
Irf4-2	1	3	MM5000006644	CTTTGAGGAATTGGTCGAGAGG
Isg20-1	1	3	MM5000023537	CACGACTTCAATGCCCTGAAGG
Isg20-2	1	3	MM5000023538	CTATGACACGTCCACAGACAGG
Itm2c-1	-1	2	MM5000015435	TACAACCATGCCCATGGACAGG
Itm2c-2	-1	2	MM5000015436	TTCCGGGAGCGGTGGCGAGGGG
Kcnn4-1	-1	2	MM5000023239	GGTGATCAAACACTTAACCAGG
Kcnn4-2	1	3	MM5000023240	CATGACTGACAACGGGCTCCGG
Kdelr1-1	1	3	MM5000007133	AACCACGACACTTTCGGGTGG
Kdelr1-2	1	3	MM5000007134	TGGCAACCACGACACTTTCGGG
Kdelr2-1	1	2	MM5000009699	GTCTTCACGACTCGCTACCTGG
Kdelr2-2	1	2	MM5000009700	AGTCAGCTCCTGTTGCACTGG
Klf2-1	1	2	MM5000030891	ACCAAGAGCTCGCACCTAAAGG
Klf2-2	1	2	MM5000030892	TCCATGGGATTGGACGGTCTGG
Klhl14-1	1	3	MM5000007399	TAATACTGAACCAGATTGCTGG
Klhl14-2	-1	3	MM5000007400	ATTGCCTCCCGGACCAGACCGG
Klhl6-1	1	4	MM5000020809	AACTCATGCATCCTGGGCTTGG
Klhl6-2	1	4	MM5000020810	TGCTCTGTCAGTGGGAGCTTGG
Krtcap2-1	1	2	MM5000008771	AGTGGCTCACCATTCAGGGGGG
Krtcap2-2	1	2	MM5000008772	CCAGCTGGCCTCCACCGAGTGG
Larp1b-1	1	4	MM5000009161	CTCAGAGTGTTATCAACCAAGG
Larp1b-2	1	4	MM5000009162	GGCTCAGTCAAGCAGCCAGAGG
Lbh-1	1	2	MM5000008509	GTCTCAGCCCCCGCAAGGATGG

Lbh-2	1	2	MM5000008510	ATGATGAACGCTCCATCCATGG
Lck-1	1	3	MM5000035623	CTTCAGAGCCATTCCGGATGGG
Lck-2	1	3	MM5000035624	GGTGGGAGAGATCCCTCATAGG
Lcp1-1	1	4	MM5000011319	CTGATGGCCACAGGTGATCTGG
Lcp1-2	-1	4	MM5000011320	TTTCTCTCACTCGGTACCCAGG
Leprotl1-1	-1	2	MM5000000877	TGTCCTTTGGAGGAGCGATCGG
Leprotl1-2	1	3	MM5000000878	TCCTTACACGCGTTGCTCATGG
Lman1-1	-1	2	MM5000016469	TGACCGGAAGAGGTGGAATTGG
Lman1-2	-1	2	MM5000016470	GAGGTGGAATTGGAGCCGATGG
Lrrc59-1	1	2	MM5000010671	CTTCCAAGGCTACGGTATTGG
Lrrc59-2	-1	2	MM5000010672	GATCCAATACCGTAGCCTTGGG
Lrrk2-1	-1	2	MM5000021623	GACAATCAACAGAGGCACGTGG
Lrrk2-2	-1	2	MM5000021624	AGTCCAGGACAATCAACAGAGG
Ly6c1-1	-1	4	MM5000012483	CCCTGCTGGTGTGCCAATCAGG
Ly6c1-2	1	4	MM5000012484	TGATGTTAGGATCCCTGATTGG
Ly6c2-1	1	3	MM5000021387	TGATGTTAGGATCCTTGATTGG
Ly6c2-2	-1	2	MM5000021388	TGCAGTGCTACGAGTGCTATGG
Ly6k-1	1	3	MM5000021751	CACTGGGACGGATTCGAGCAGG
Ly6k-2	1	4	MM5000021752	TGGTGGATTAGTGCTAGGCGGG
Ly86-1	-1	2	MM5000026279	AGATTTGATTGGATCTGCTTGG
Ly86-2	1	2	MM5000026280	CAAATCTCAACATTAGATTTGG
Maged1-1	1	3	MM5000005185	GCTTCAGAGATACCTGCGCTGG
Maged1-2	1	3	MM5000005186	GTCCCTACGTCAGCCATTTTGG
Magt1-1	1	2	MM5000013529	CAACTGAGTAATTTCTCGGTGG
Magt1-2	1	2	MM5000013530	TGGAGCTTTAACAAGACGACGG
Man1b1-1	1	2	MM5000002131	GCAGCTTTCGAGGCTACAGCGG
Man1b1-2	1	2	MM5000002132	TCTTCATACGGCTGACCAGTGG
Manea-1	-1	3	MM5000030699	GGCGAAGCTACTGATCACTTGG
Manea-2	1	3	MM5000030700	GTCATCCCTTGAATCAGGTGGG
March1-1	-1	6	MM5000022409	GCGACAGGGTGTGATGAGTGGG
March1-2	-1	6	MM5000022410	TGAGCTCTTGATCCATTGGTGG
Marcksl1-1	-1	2	MM5000010125	CATCTGTTCCGTTACGGGTGG
Marcksl1-2	-1	2	MM5000010126	CCTCATCTGTTCCGTTACGGG
Mars-1	1	2	MM5000030717	CCTTAGGCCGGGTAAGGAATGG
Mars-2	-1	2	MM5000030718	CCATTCTTACCCGGCCTAAGG
Mcdf2-1	1	3	MM5000001623	ACATCAGCCCCGTGGTCATGGG
Mcdf2-2	-1	3	MM5000001624	GATGTCCATCATGGCAGCGTGG
Mettl9-1	1	2	MM5000007105	TTCAGAGTTATCTTGACCAAGG
Mettl9-2	-1	2	MM5000007106	TACAAAAGAATGATAGAGTTGG
Mgat2-1	1	1	MM5000021229	AAAGGAAGAGCGACGCCCTTGG
Mgat2-2	1	1	MM5000021230	CAACCTAACGCTGCGGTACCGG
Mknk2-1	1	3	MM5000030625	TTGGTGATGAGATTGACACAGG

Mknk2-2	-1	4	MM5000030626	GCTGGGCCACATCCGCAGCAGG
Mnda-1	1	5	MM5000025855	CTCTCAAAGTAATTGGATATGG
Mnda-2	1	5	MM5000025856	CATTTCTGGTAATACAGTTGGG
Mrp63-1	-1	2	MM5000024767	CTTGGAAGACACGGTACGCGG
Mrp63-2	1	2	MM5000024768	ACCGTGTCTTTCCAAGCGAAGG
Ms4a1-1	-1	3	MM5000040433	GACTGCTGATGATCCCCACAGG
Ms4a1-2	1	3	MM5000040434	CAGTCCCCCAGGGTAATATGG
Ms4a4c-1	-1	3	MM5000040439	AATTATTATTCCGAAGCTGAGG
Ms4a4c-2	1	3	MM5000040440	GGTCTTAATGGCTCCAATTTGG
Ms4a6c-1	-1	4	MM5000040443	GACTTTCTCTCCGTAATGATGG
Ms4a6c-2	-1	2	MM5000040444	GCTGGGACTCGTCTTTTGGGG
Msn-1	-1	2	MM5000005353	TCCAGCTCTGCATCCATGGTGG
Msn-2	1	3	MM5000005354	GGTGAAACTATTGTTTGAGG
Mt1-1	-1	2	MM5000026455	TTGCAGTTCTTGACAGCGCAGG
Mt1-2	-1	3	MM5000026456	CAGCACGTGCACTTGTCGCGG
Mt2-1	-1	2	MM5000001063	CAGGCGCCAGCGCAGGAGCAGG
Mt2-2	1	3	MM5000001064	GGCTGTGCGAAGTGCTCCAGG
Mtdh-1	1	2	MM5000021157	CGGACTGTTGAAGTACCCGAGG
Mtdh-2	-1	2	MM5000021158	GTTTTGCAGTTATACTTCGGGG
Mthfd2-1	1	2	MM5000025137	ACTTCTTGCTTGATCTGCTGGG
Mthfd2-2	1	4	MM5000025138	TGCTGGCAGCATAGAGTACTGG
Mzb1-1	1	2	MM5000030407	CCTGCAATCCACTGGCGTCTGG
Mzb1-2	-1	2	MM5000030408	CCAGACGCCAGTGGATTGCAGG
Nans-1	1	2	MM5000009131	TTCAGCCACGACCAGTACAAGG
Nans-2	-1	2	MM5000009132	CAGCTCCTTGACTGGTCGTGG
Nars-1	-1	2	MM5000002611	GAAGGAAATGACGCAACCGGGG
Nars-2	-1	2	MM5000002612	GAGAAGGAAATGACGCAACCGG
Nbeal2-1	-1	2	MM5000030447	ACTATCTCGTTATCCTCCCTGG
Nbeal2-2	1	3	MM5000030448	CAACAGCAGGGCTTGATCCAGG
Ncf1-1	1	2	MM5000019211	GTAGACCACCTTCTCCGACAGG
Ncf1-2	1	2	MM5000019212	CAGGTCTGCCACTTAACCAGG
Nckipsd-1	-1	4	MM5000030453	TGAGATCACCAGGGCCTCACGG
Nckipsd-2	1	4	MM5000030454	GTGAAGCGCCGAGACCGTGAGG
Neil1-1	1	2	MM5000030313	TCACAGATGGGCCGGTCAAAGG
Neil1-2	-1	2	MM5000030314	TGGCATTGGCAACTATCTGCGG
Neur13-1	-1	2	MM5000040551	TCTTCGCCAAGGTCAACGCTGG
Neur13-2	1	2	MM5000040552	GTCTTTGCGCAGCAAGAGGGGG
Nfkbid-1	1	4	MM5000015325	GAGTCTCTAGTCTCCCCTCAGG
Nfkbid-2	1	4	MM5000015326	ACAGCCCGAGCTCACATATTGG
Notch2-1	-1	2	MM5000024905	AAGCCTGTGCCGTTGTGGTAGG
Notch2-2	-1	2	MM5000024906	GCAGAAGCCTGTGCCGTTGTGG
Nrm-1	1	2	MM5000034673	CTTCAGAGGTCGCTCTATGTGG

Nrm-2	-1	2	MM5000034674	CACTGCCTCAGTCGCCATAAGG
Nuak2-1	1	2	MM5000015425	GGATCTGCTGCACATACGGAGG
Nuak2-2	1	4	MM5000015426	TTACATCAGTGAGCGGCCACGG
Nucb1-1	1	3	MM5000015197	GAGGTACCGGTGGTAGTACAGG
Nucb1-2	-1	3	MM5000015198	TACTACCACCGGTACCTCCAGG
Nudfa1-1	1	2	MM5000005139	GCGTTCCATCAGATACCACTGG
Nudfa1-2	-1	2	MM5000005140	AACGCGATAGACGTATCTCTGG
Nudt22-1	1	3	MM5000001621	TCGTACCACCAGCAACCCGGGG
Nudt22-2	1	3	MM5000001622	GGGGAGGTCCTTGTGCCGGGGG
Nxpe2-1	1	2	MM5000030165	AAAGATAATTAACACTATCAGG
Nxpe2-2	-1	3	MM5000030166	GACCAGCTGAACGTCTTACTGG
Oosp1-1	1	2	MM5000029781	TGGCCAGGTAGTGGTCACAGGG
Oosp1-2	-1	2	MM5000029782	TCTACCGGACTTACTCCTGCGG
Os9-1	1	2	MM5000015421	AACCACCACGTCTGAAGCTTGG
Os9-2	1	2	MM5000015422	CTCCTCCTCTCTTTCACGTTGG
Oxct1-1	-1	4	MM5000021335	CTGCTTGGATCGAAGTAAAAGG
Oxct1-2	1	5	MM5000021336	GGGTATGGGACTCTGGTACAGG
P4hb-1	-1	9	MM5000010855	CAAGCAGCTAGCCCCCATTTGG
P4hb-2	-1	9	MM5000010856	GCCCCCATTTGGGATAAACTGG
Parp8-1	-1	3	MM5000011509	CCTTTACTTTTACCTACGTTGG
Parp8-2	1	4	MM5000011510	TCTGAGACGTGGACGGAGTAGG
Pax5-1	-1	2	MM5000024621	CCCAGATGTAGTCCGCCAAAGG
Pax5-2	1	2	MM5000024622	CCTTTGGCGGACTACATCTGGG
Pck-1	-1	2	MM5000029749	GGGTTTGGATGCTACGGCACGG
Pck-2	1	2	MM5000029750	CACATCTGTGATGGGACCGAGG
Pde2a-1	-1	2	MM5000003175	CGGCGGCTCGTCCGGCTTGAGG
Pde2a-2	-1	2	MM5000003176	GCTGCGGCGGCGGCTCGTCCGG
Pdia6-1	1	2	MM5000017247	CCTTCTTTCTAGCAGTCAGCGG
Pdia6-2	-1	3	MM5000017248	CCATTCTGGTGTTAACCTCTGG
Pear1-1	1	5	MM5000029625	TGGGACTCCTTAGTGGTCGTGG
Pear1-2	-1	6	MM5000029626	TACCGGACTGTGTACCGTCAGG
Pgap1-1	-1	2	MM5000037836	AGTTATATCTTTACGGAGAAGG
Pgap1-2	1	2	MM5000037837	AAAGATATAACTCATATGCTGG
Pik3c2b-1	1	2	MM5000015061	TGTCTTCCACTCAGGGCAATGG
Pik3c2b-2	1	2	MM5000015062	TCTTCCACTCAGGGCAATGGGG
Pik3ip1-1	1	2	MM5000004425	TCAACTGGTTGGCCGCGCAAGG
Pik3ip1-2	-1	2	MM5000004426	GGGCTCGGTGAGCGACTCGCGG
Plcb2-1	-1	2	MM5000007781	TCTCCTGTTATCCTCCGTGTGG
Plcb2-2	1	2	MM5000007782	GATCCACACGGAGGATAACAGG
Plekhg2-1	1	4	MM5000034499	TCATAGATGTCCTCGATGTTGG
Plekhg2-2	-1	4	MM5000034500	ACACTGTTTGCCAACATCGAGG
Pold1-1	-1	2	MM5000000431	TGGATTGTAAGCGGCGACAAGG

Pold1-2	1	2	MM5000000432	GGTTCGCCTCAAACCTGCGACGG
Pold4-1	-1	2	MM5000027543	GTACAAGGCCCATACTGCCAGG
Pold4-2	1	2	MM5000027544	TTGACCTGGCCTGGCAGTATGG
Pon2-1	-1	4	MM5000007267	TCTCAAAGACGAGAGACCGAGG
Pon2-2	-1	4	MM5000007268	GCTTTGCACCGGATAAGCCTGG
Pon3-1	1	3	MM5000010633	AGATAAAAGCCAGCCCCGCTAGG
Pon3-2	1	4	MM5000010634	GTTTGTCCGGTGCAAACGCTGG
Ppapdc1b-1	1	2	MM5000022439	TACAGGAACCCGTACGTGGAGG
Ppapdc1b-2	1	2	MM5000022440	CTCTACAGGAACCCGTACGTGG
Pqlc3-1	1	2	MM5000021299	CTGATAACGAAGGAAGACCAGG
Pqlc3-2	-1	4	MM5000021300	CAGAAATGGATCATCGACTTGG
Praf2-1	1	2	MM5000012811	CTCCTGAGTGCACCTGGTAGTGG
Praf2-2	1	2	MM5000012812	CTCTTCATTCTCTGGGCCGTGG
Prdm1-1	-1	4	MM5000029035	GGCTCCACTACCTTATCCTGG
Prdm1-2	-1	4	MM5000029036	TTGTCGGGACTTTGCGGAGAGG
Prdx4-1	-1	2	MM5000013411	AGCCAGCACCTTATTGGGAAGG
Prdx4-2	-1	2	MM5000013412	GTGATTAACGGAGAATTCAAGG
Prg2-1	-1	3	MM5000007561	GCATTCAGCCCTCCTGACTAGG
Prg2-2	-1	3	MM5000007562	TTCAGCCCTCCTGACTAGGAGG
Prkcb-1	-1	3	MM5000038099	CGGGCCCTTGTCCGCACCGGGG
Prkcb-2	-1	3	MM5000038100	CCCTTGTCCGCACCGGGGCAGG
Prmt7-1	-1	3	MM5000026475	TCATGTAGCATGTCTGGCATAGG
Prmt7-2	-1	4	MM5000026476	CACAGCTGCCCCGATACCCTGG
Prrc1-1	1	3	MM5000028941	CAACCTACGACATCACGAGGGG
Prrc1-2	-1	3	MM5000028942	ACTCCATTCCCACACGTTGGG
Pstpip1-1	1	2	MM5000039317	GGGACTTCACGGCCACACAGG
Pstpip1-2	1	3	MM5000039318	TGCAGATTGCACGCAAGGCTGG
Pxdc1-1	-1	2	MM5000000757	AATGAGGTGCGAGAAGCTGCTGG
Pxdc1-2	1	2	MM5000000758	ATGTCATGTGCACCCTTGATGG
Pxk-1	1	2	MM5000006985	ATATCATCCGGGTGCAAAGAGG
Pxk-2	1	4	MM5000006986	CCGTGAGTTCATCGCTGAGAGG
Pycr1-1	-1	2	MM5000038595	ATGGACCAAGCTACGGTCTCGG
Pycr1-2	1	2	MM5000038596	CCGTAGCTTGGTCCATGTCTGG
Qpctl-1	1	2	MM5000001225	GATTGCCAGAACTACCCGGGGG
Qpctl-2	1	2	MM5000001226	TGAAAGGCTTCCGATCAGCGGG
Rapgef3-1	-1	22	MM5000028755	CTTTCTTTGCGGTCTGTTTGG
Rapgef3-2	1	24	MM5000028756	GAGAGCCAATCGGTACACTCGG
Rasgef1b-1	1	4	MM5000010927	CCTCATCATTCTCTCGTCCCGG
Rasgef1b-2	-1	4	MM5000010928	CCTCAAGGACCTGGCGCACAGG
Rasgrp1-1	-1	2	MM5000028765	ATGGTGTCCCTGGGACATTTGG
Rasgrp1-2	1	2	MM5000028766	GACACCATCATTTCGGAAGTGGG
Rbm38-1	1	3	MM5000038201	CCTATCATCGATGGTCGCAAGG

Rbm38-2	1	3	MM5000038202	CGCAAGGCCAATGTGAACCTGG
Rcsd1-1	1	6	MM5000039369	ACAGTCCGACTGAGACCTTCGG
Rcsd1-2	1	6	MM5000039370	TGGTTGAGTTGAGGGGACCCGG
Rel-1	-1	2	MM5000040687	AGTGTGAAGGGCGATCAGCAGG
Rel-2	-1	2	MM5000040688	TCAGCAGGTAGCATCCCAGGGG
Reln-1	-1	2	MM5000028803	TTGGAGGCTCCAGCGCCTTTGG
Reln-2	-1	3	MM5000028804	TCATGTCCGACCACCAGTTTGG
Rexo2-1	1	2	MM5000005191	AAGATCAGAGTCAGTTATCAGG
Rexo2-2	-1	2	MM5000005192	AAGGATCAGATTATTGAGATGG
Rgcc-1	1	2	MM5000028637	TCGAACTCGCACAGCACGTCCG
Rgcc-2	1	2	MM5000028638	ATAGTGGAAGTGCGCTCGTGG
Rhob-1	1	1	MM5000021001	ATGACGTCGGTGTCCGGATAGG
Rhob-2	1	1	MM5000021002	TAAGGATGACGTCGGTGTCCGG
Rhobtb1-1	1	4	MM5000024307	AAGACAGGCGGTTTCGCATATGG
Rhobtb1-2	-1	4	MM5000024308	ACTTCATCGACGACATCCCTGG
Rin3-1	1	3	MM5000040459	GACAACAACCTGAAGCAATTGG
Rin3-2	-1	3	MM5000040460	ACACAGCACCAATTGCTTCAGG
Ripk3-1	1	4	MM5000000911	TCTGGGTCCAAGTACGCTAGGG
Ripk3-2	-1	4	MM5000000912	TTTGGCCTGTCCACGTTTCAGG
Rnf130-1	1	2	MM5000014553	GGATCGCCTTGCTACAAAGAGG
Rnf130-2	-1	2	MM5000014554	GGACAAAGAACCGTGTTTGAGG
Rpl10-1	1	6	MM5000004831	ATCCCCAATCGTGGTCCTCTGG
Rpl10-2	-1	6	MM5000004832	GTCCAGAGGACCACGATTGGGG
Rpl15-1	-1	3	MM5000021327	ACAGGATTTCGTGTCCGCCGTGG
Rpl15-2	1	3	MM5000021328	GCGTTTACGACCACCACGGCGG
Rpl23a-1	1	3	MM5000025493	TTCTTCATGGCTGACTCCGTGG
Rpl23a-2	1	3	MM5000025494	GGCTGACTCCGTGGTCAGTGGG
Rpn1-1	-1	2	MM5000007797	TTTGGTTTCTCGTACTTCTAGG
Rpn1-2	1	3	MM5000007798	TCCAAGATCTCGGTCGTTGTGG
Rpn2-1	1	7	MM5000011791	CTCTCAGAAGCCTTCAGTGTGG
Rpn2-2	1	7	MM5000011792	AATCGCTACCACGTACCAGTGG
Rps27l-1	1	2	MM5000013147	CTGTTACACCCTTCCTTGAGG
Rps27l-2	1	2	MM5000013148	AAACATAAGAAGAAACGGCTGG
Rps6-1	-1	3	MM5000023803	TCTGTTTCGTGGATGCATTGTGG
Rps6-2	1	3	MM5000023804	GTTCTCCTTGGCCTGTAACAGG
Rps6ka5-1	-1	2	MM5000028575	ACAGGACACGCAGAGAAGGTGG
Rps6ka5-2	-1	2	MM5000028576	TTAACAGGACACGCAGAGAAGG
S1pr1-1	1	2	MM5000024895	CCTGCTAATAGGTCCGAGAGGG
S1pr1-2	1	2	MM5000024896	GATGTTCTTGCGGAAGGTCAGG
Samd9l-1	-1	2	MM5000005217	GGCTGTGGAGGTACTACGTTGG
Samd9l-2	1	2	MM5000005218	AACGGGAATGAAGTCCTCGTGG
Sbk1-1	-1	3	MM5000019803	GATGAAGGGGCTAGACGACAGG

Sbk1-2	1	3	MM5000019804	GACGTGGTCTTCGAGACCGAGG
Scd1-1	1	3	MM5000035603	GTCACATCTCATCCGCACGGGG
Scd1-1	1	2	MM5000012175	GTGGTGGTCGTGTAAGAACTGG
Scd1-2	-1	3	MM5000035604	CTGACTGTTGAGGCCCGTTGGG
Scd1-2	1	2	MM5000012176	CTCGTTCATTTCCGGAGGGAGG
Scml4-1	1	5	MM5000004217	GTCTTCTCCCTCGTCAAGCAGG
Scml4-2	-1	5	MM5000004218	CGGTATCCCTGCTTGACGAGGG
Scn4a-1	1	2	MM5000039785	GTGGCAGAGAATCGGAAGATGG
Scn4a-2	1	2	MM5000039786	CACCTTGATAGCCACCCGCCGG
Sdf2l1-1	1	2	MM5000021145	GTTGTTGGATAGCGGCGACGGG
Sdf2l1-2	-1	2	MM5000021146	CAACAGTCGGTAACCGGCGTGG
Sec11c-1	-1	2	MM5000039481	AGCGCAGAAGACACGATCATGG
Sec11c-2	-1	2	MM5000039482	GCTGCCCCGTGAGAACAATCAGG
Sec22b-1	1	2	MM5000009257	TCCCCTACCCGATGTACCTTGG
Sec22b-2	-1	2	MM5000009258	CTTCCAAGGTACATCGGGTAGG
Sec24a-1	1	2	MM5000004049	TTGGACGAGGGTAGGATCCTGG
Sec24a-2	1	2	MM5000004050	CTTTGCTGGCATTGACGAGGG
Sec24d-1	-1	3	MM5000010497	CCTGGAGTATGGGCTCCATTGG
Sec24d-2	1	3	MM5000010498	CCAATGGAGCCCATACTCCAGG
Sec61a1-1	-1	4	MM5000007697	CTCAGCTGACCCGTTTCTACTGG
Sec61a1-2	1	4	MM5000007698	CACTCGCATCCAGTAGAACGGG
Sec63-1	1	2	MM5000035507	ATGTATGTGGTATCGTTTACGG
Sec63-2	-1	3	MM5000035508	TGATATTCTCGGTCTGTTTTGG
Sel1l-1	1	3	MM5000011363	CCAGCAACTACTTTGCCCGTGG
Sel1l-2	1	3	MM5000011364	GCAACTACTTTGCCCGTGGTGG
Selk-1	1	4	MM5000004207	GTAGGATCAGTCACCTTCGTGG
Selk-2	-1	4	MM5000004208	AGGAGGGCTGGGGCCACGAAGG
Sell-1	1	3	MM5000006259	TCAAGAGGGAACGAGACTCTGG
Sell-2	1	3	MM5000006260	GACGCCTGTCACAAACGAAAGG
Selm-1	1	2	MM5000004107	TGACAGTTGAATCGCCTAAAGG
Selm-2	-1	4	MM5000004108	GCTTAACAGCACGAGTTCGGGG
Sepp1-1	-1	3	MM5000024521	GCTTTCTAGTTTTATGCGCAGG
Sepp1-2	1	3	MM5000024522	AGAAGATGGCATAGATGTCTGG
Serinc5-1	-1	2	MM5000011279	CCGAGACTGTCGGAACCTAGGG
Serinc5-2	-1	2	MM5000011280	CCAGCAGATCCGCAACAGCAGG
Serpina3f-1	1	4	MM5000006551	TGGGAGATGCCCTTTGATCCGG
Serpina3f-2	1	4	MM5000006552	ATGTTTCATCCTCCCGACCAGG
Serpinb1a-1	1	3	MM5000021183	CAGGCTTTGGAAGCGTGAATGG
Serpinb1a-2	-1	3	MM5000021184	ATGCTGAAGTGAGCAAACGTGG
Sesn3-1	1	2	MM5000013541	TGTCTCAACCCCTGACAAGAGG
Sesn3-2	-1	2	MM5000013542	TGTCAGGGGTTGAGACACTCGG
Sh3bp5-1	1	3	MM5000035537	CAGGACAGATCGGAACCTTCTGG

Sh3bp5-2	1	3	MM5000035538	TTTCACCGTTGCCTCGACCAGG
Sh3pxd2a-1	-1	2	MM5000007903	CTCCCAGACTATCTACCGGAGG
Sh3pxd2a-2	-1	2	MM5000007904	CACCTCCCAGACTATCTACCGG
Sil1-1	-1	3	MM5000005649	CACCAGGAATCAGATACAAAGG
Sil1-2	1	4	MM5000005650	TGTAGGTTTCATTTCGCACATGGG
Sipa1-1	-1	5	MM5000008495	CACCAGAACTTCTTTGGGCTGG
Sipa1-2	1	5	MM5000008496	CGTGGTCCGCACGATGACCCGG
Sipa1l2-1	-1	2	MM5000026231	AGGGTCGCAGTTCAACTACAGG
Sipa1l2-2	-1	2	MM5000026232	TGTGGCGGTCAGCATCCGGAGG
Slamf1-1	1	2	MM5000006065	CCTGAAGATCCTCGGGAACAGG
Slamf1-2	1	2	MM5000006066	AGCTATCCAGATCACCTGGAGG
Slamf7-1	1	2	MM5000006187	GACATGCAGCACATACTCCTGG
Slamf7-2	-1	2	MM5000006188	ATTTACAGTACATCGAGTCAGG
Slc12a4-1	1	2	MM5000039507	TCCTCTGGAGGCGTCCAAAGGG
Slc12a4-2	-1	2	MM5000039508	GACTACTATGACCGGAACTTGG
Slc33a1-1	1	3	MM5000038617	TGGGACAGCTAGTAAGGCCAGG
Slc33a1-2	-1	3	MM5000038618	GCCTTACTAGCTGTCCCAATGG
Slc35b1-1	1	2	MM5000014237	TTACACCTTTGCCTTAACCTTGG
Slc35b1-2	1	3	MM5000014238	TGCCAGGGTGGATCGCACTCGG
Slc37a2-1	1	3	MM5000039547	GTGGTATCATTGAGATCGTGGG
Slc37a2-2	1	3	MM5000039548	GATCGTGGGTGTCATTAACAGG
Slc39a11-1	-1	3	MM5000017435	CGGATCTTAGACGGAAGCCTGG
Slc39a11-2	-1	4	MM5000017436	CATGTTAGCGGCTTCCTACTGG
Slc39a4-1	-1	3	MM5000035705	CAGGGGTTTCCAAAAGCGCCGG
Slc39a4-2	1	3	MM5000035706	AAAGTACTGAGGGCTAGGCAGG
Slc39a7-1	-1	2	MM5000008231	GCTCAGTTTTGCTTCCGGGGGG
Slc39a7-2	-1	2	MM5000008232	CTGCTCAGTTTTGCTTCCGGGG
Slc3a2-1	1	3	MM5000012515	GTGAATTGGGCCTAACACCAGG
Slc3a2-2	1	4	MM5000012516	AGGGAGGAACCACGCATTCTGG
Slc44a1-1	1	7	MM5000013501	GATCCTGATGGTGATTATCAGG
Slc44a1-2	-1	7	MM5000013502	ATACCTGATAATCACCATCAGG
Slc44a2-1	-1	2	MM5000013373	AGGTGGGGTTCGTATTTCTGAGG
Slc44a2-2	1	3	MM5000013374	CTGGCCATCGTAGGCTATGTGG
Slc7a5-1	-1	2	MM5000022229	AAGGCTGCGACCCGTGTGCAGG
Slc7a5-2	-1	2	MM5000022230	GTGAACTGCTACAGCGTAAAGG
Slfn5-1	-1	5	MM5000001133	GGTCCTTGTAGACGCTCTCTGG
Slfn5-2	1	5	MM5000001134	ACTCCAGAGAGCGTCTACAAGG
Slpi-1	1	2	MM5000037939	CCCAGTCAGTACGGCATTGTGG
Slpi-2	-1	2	MM5000037940	GCCACAATGCCGTAAGTACTGG
Snd1-1	-1	2	MM5000025139	AATTATGGCGCACCCAGAGAGG
Snd1-2	-1	2	MM5000025140	GGCTGCCCTCGGACAATTATGG
Snx2-1	1	3	MM5000012249	TCCTCCGAGCCGTCTCCTGCGG

Snx2-2	-1	3	MM5000012250	GAGTGACAGGGGTGACCGCAGG
Snx9-1	1	2	MM5000001693	ATGAGCTGACCGTTACTGAAGG
Snx9-2	1	3	MM5000001694	CTTGTTCTACTGACTACGTGG
Sorl1-1	1	2	MM5000013745	CTTGGGCCTTGCCAACGCCAGG
Sorl1-2	-1	3	MM5000013746	GAGAAGCTGAACTTTGGCGTGG
Spcs1-1	-1	2	MM5000036513	CTAGCTGAACAGATGTTTCAGG
Spcs1-2	-1	2	MM5000036514	AGCTGAACAGATGTTTCAGGGG
Spcs2-1	1	5	MM5000000661	CATGACCAATGTCCCGCTGTGG
Spcs2-2	-1	3	MM5000000662	TCCTGCTTTTTTGCCATCGTGG
Spcs3-1	-1	4	MM5000000663	GGGACAAAATTGTTTTGAGAGG
Spcs3-2	-1	5	MM5000000664	GGAACGTTGTACCAAACGCTGG
Spib-1	1	4	MM5000001329	AAGGCTTCATAGGGAGCGATGG
Spib-2	-1	4	MM5000001330	CTCCACCTATAGCCCCATGGGG
Spint2-1	1	5	MM5000022391	TCATAGTACCAGCGAGGGAAGG
Spint2-2	1	5	MM5000022392	CGGCCCACTGACGGCCTTCGGG
Spns2-1	-1	2	MM5000001265	ACTTTGGGGTCAAGGACCGGGG
Spns2-2	1	2	MM5000001266	CTTGACCCCAAAGTGCTGCTGG
Spon1-1	-1	3	MM5000003855	TGCTTTCAGTGACGGCCACAGG
Spon1-2	1	3	MM5000003856	CACTGAAAGCACCCCTCGGAGG
Srp54a-1	1	2	MM5000038535	GAAGTATGTACAGCATTATTGG
Srp54a-2	1	4	MM5000038536	AAAGCGTGGACACCGACTAAGG
Srp54c-1	1	2	MM5000037613	GAAGTATGTACAGCATTATTGG
Srp54c-2	1	4	MM5000037614	AAAGCGTGGACACCGACTAAGG
Srp3-1	-1	2	MM5000028369	GGAGTCCCTGCAGCAAACGAGG
Srp3-2	-1	2	MM5000028370	CCTGCAGCAAACGAGGTGCTGG
Srpr-1	1	3	MM5000013007	TGATGTGCACCGACTGTTCCGG
Srpr-2	-1	3	MM5000013008	CTCTGTGCGATACTTATCCCGG
Srprb-1	1	3	MM5000001717	ATGGCAGAACTATCGGTAATGG
Srprb-2	1	3	MM5000001718	TTGTATATGGCAGAACTATCGG
Ssr1-1	1	4	MM5000011485	TCCTGAGGATAACGGAACGAGG
Ssr1-2	-1	4	MM5000011486	GCCTCGTTCCGTTATCCTCAGG
Ssr2-1	-1	2	MM5000010149	AGACAATGCCGAAGTCTTCTGG
Ssr2-2	1	2	MM5000010150	AAGACTTCGGCATTGTCTCCGG
Ssr3-1	1	2	MM5000010383	TTTACATTCTTGACGCGAAGG
Ssr3-2	-1	2	MM5000010384	TATTGGAGAATATGGCATATGG
Ssr4-1	1	3	MM5000013559	CTCTTTATGCCGACGTTAGTGG
Ssr4-2	-1	3	MM5000013560	AATTGTTTTCCACTAACGTCGG
St6gal1-1	-1	5	MM5000003943	TGAGAGTTCACTAGGCGGATGG
St6gal1-2	1	5	MM5000003944	TAATCATGATGCGGTCCTGAGG
Stap1-1	1	2	MM5000022103	ACTATTGGACGGAGCTGAGAGG
Stap1-2	1	3	MM5000022104	TAGACCTCGTGTGCCTTACTGG
Stk10-1	-1	2	MM5000001469	GAGCTTCACGATATACGGGTGG

Stk10-2	1	2	MM5000001470	CCGTATATCGTGAAGCTCCTGG
Stk17b-1	1	3	MM5000015409	CTCATGCAGAATTTCCGCCCGG
Stk17b-2	-1	3	MM5000015410	GAGGTCTACGAAAATGCAACGG
Stt3a-1	-1	4	MM5000004963	ACCCTTTGGGCCGAATCATTGG
Stt3a-2	-1	4	MM5000004964	CTGTTTTGATGACCGGGCTTGG
Sumo2-1	-1	4	MM5000010721	TGTTCCAGCAGCAGACTGGAGG
Sumo2-2	1	4	MM5000010722	GACACCTCCAGTCTGCTGCTGG
Surf4-1	1	5	MM5000012379	CAAACCTCCTCCTAAAGCCAGG
Surf4-2	1	5	MM5000012380	GGAGCTCTCACGCATGTTGGG
Susd3-1	-1	2	MM5000001507	TGCAAGTCGTCCGTGGAGATGG
Susd3-2	-1	2	MM5000001508	GAATGGGAGCACCGTTGACTGG
Tapbpl-1	-1	2	MM5000036455	TGACAGAAGACAGGCACCGCGG
Tapbpl-2	-1	2	MM5000036456	ACAGAAGACAGGCACCGCGGGG
Tigit-1	-1	2	MM5000018421	TCAGTCTTCAGTGATCGGGTGG
Tigit-2	1	2	MM5000018422	TGCTGCTTCCAGTCGACTTGGG
Tm6sf1-1	1	4	MM5000026481	CGTATCTGAACACGGCCTATGG
Tm6sf1-2	-1	4	MM5000026482	CATAGGCCGTGTTTCAGATACGG
Tmed2-1	-1	2	MM5000010057	GCAAATGTGTACTTCCCGCTGG
Tmed2-2	-1	2	MM5000010058	GGAGCCTCCCCAATGTCAATGG
Tmed3-1	-1	2	MM5000005363	ACTGGAGGCCACTACGATGTGG
Tmed3-2	-1	2	MM5000005364	CAGGGGCAACGTCATCTACAGG
Tmed9-1	1	2	MM5000017219	GGGCTTGGCATGTTCTAGAGG
Tmed9-2	1	2	MM5000017220	GTAGAGGTAAAAGACCCCGAGG
Tmem176a-1	1	2	MM5000039629	ACATGGAGACTGCAGTCGTCGG
Tmem176a-2	1	2	MM5000039630	ATCCACCAGGAGTCTGCTCTGG
Tmem176b-1	-1	3	MM5000036195	ATGTCTCTACTTCGGGCCTTGG
Tmem176b-2	-1	3	MM5000036196	TGGCTGTGCTTTCTGGTCGGGG
Tmem184b-1	1	3	MM5000018547	CGATGTGGCGCTGCTCATTGGG
Tmem184b-2	-1	3	MM5000018548	ACCAGTACTACGTGTACTTCGG
Tmem214-1	-1	2	MM5000011133	GTCCTCCAGGCGTCGGAATGGG
Tmem214-2	1	2	MM5000011134	CAGGGCCCATTCCGACGCCTGG
Tmem243-1	-1	4	MM5000021939	TGGTTTGGGAGGTAGTTGAGGG
Tmem243-2	1	5	MM5000021940	AACTTGTACTTCCATGATGTGG
Tmem248-1	-1	8	MM5000022004	GAAGGCAGGGGCTCAGGCGTGG
Tmem248-2	1	3	MM5000022005	CCATCCTCGGGCATCAGATTGG
Tmem258-1	-1	3	MM5000036217	TCGCGTGTGTACTTGGTAGAGG
Tmem258-2	-1	3	MM5000036218	TAAATATCGCGTGTGTAATTGG
Tmem39a-1	-1	3	MM5000028201	AGAACAGGCTCCCATCTACTGG
Tmem39a-2	-1	3	MM5000028202	GGATTTGGCAATGACGAACAGG
Tmem66-1	-1	2	MM5000020137	GTGTAGCGGTCGGAGTAGAGGG
Tmem66-2	-1	2	MM5000020138	TGTATCACCTGGGGGTATAGG
Tnfaip8l2-1	1	2	MM5000036233	CCGAAGCTAAGTGCGGTCATGG

Tnfaip8l2-2	1	2	MM5000036234	CGGCTGTGCGTGATTCTTTGG
Tnfrsf17-1	1	2	MM5000019261	TCAGTGAAAGGGACGTACACGG
Tnfrsf17-2	1	2	MM5000019262	AGGGACGTACACGGTGCTCTGG
Tns3-1	1	8	MM5000028237	TGGATGACGACCACATGCTGGG
Tns3-2	1	8	MM5000028238	GATGACGACCACATGCTGGGGG
Top1-1	1	3	MM5000011695	GATCGAGAACACCGGCATAAGG
Top1-2	-1	3	MM5000011696	CTTCTTGCTCCTTATGCCGG
Tpst1-1	1	3	MM5000009965	GTCATCAAACCCGTCAACGTGG
Tpst1-2	1	3	MM5000009966	CATCAAACCCGTCAACGTGGGG
Trabd-1	-1	4	MM5000019285	GTCAGTGTACGTGGAAGGTTGG
Trabd-2	-1	4	MM5000019286	CTAGCTGGGTCACTGTACGTGG
Traf5-1	-1	3	MM5000019433	ATCTGCCCCGTAGACAAGGAGG
Traf5-2	1	3	MM5000019434	CCTTGTCTACCGGGCAGATCGG
Tram2-1	1	2	MM5000015587	GCACACTAATGTTATACTGAGG
Tram2-2	1	3	MM5000015588	GATGGTGACCAGGTCTTTGGG
Trem12-1	1	2	MM5000019243	GTGCTCCTACAAGAACCGCCGG
Trem12-2	1	2	MM5000019244	TCAGGACTCCGGACGATACTGG
Trim34a-1	1	2	MM5000028265	ACAGGTCTGACGAATATACAGG
Trim34a-2	-1	2	MM5000028266	TTCTTCTTCGTGATGAGACAGG
Trp53inp1-1	-1	3	MM5000009445	CTTGTTTCCACCTTGATAGTGG
Trp53inp1-2	1	3	MM5000009446	GGATTAACCACTATCAAGGTGG
Ttyh3-1	1	2	MM5000009811	GTGGTGATGATGACGCACCAGG
Ttyh3-2	1	3	MM5000009812	TTGGCATGGCGTAGTGAGTAGG
Tvp23b-1	-1	2	MM5000019443	TTGGATTTTCTTGGTCTATTGG
Tvp23b-2	1	3	MM5000019444	GCTGCTGTCATGCGACTTTTGG
Txndc11-1	1	2	MM5000012049	TCAAGGACCGGAGATCTCGAGG
Txndc11-2	-1	2	MM5000012050	TATGCTGACCATGTCCGCCAGG
Txndc5-1	1	4	MM5000021539	CTCTGGGTGGTTCGGCTTCCGG
Txndc5-2	1	5	MM5000021540	CTTTGCAGTGACCGCACACCGG
Uba-2	1	3	MM5000022642	CCTACTATTGCTACAGCGTAGG
Uba5-1	-1	2	MM5000022641	CTGATGGCATTGAAACGGATGG
Ubb-1	-1	2	MM5000038817	CCATCTTCTAGCTGCTTGCCGG
Ubb-2	1	2	MM5000038818	TGGTCCTTCGCCTGAGAGGTGG
Ube2j1-1	1	2	MM5000010073	CATTACCACGCTCAGCCTCTGG
Ube2j1-2	-1	3	MM5000010074	TGGCAGTACTATGCGTCCATGG
Ubxn4-1	1	4	MM5000036183	CCCTTTGGAAGTAATAGCGGGG
Ubxn4-2	-1	4	MM5000036184	TGGATGGAACACATACTACAGG
Ufc1-1	-1	6	MM5000015255	CTAGCTCATCTCATGGCCCTGG
Ufc1-2	1	6	MM5000015256	CCAAACTTAGGCACATTCCTGG
Uso1-1	-1	2	MM5000009695	CGGTCATCCAGTAACGTAGAGG
Uso1-2	1	2	MM5000009696	GTAGCTTCCTCTACGTTACTGG
Vcp-1	1	6	MM5000036725	CAATCGCCTTAAAGAGCGCAGG

Vcp-2	-1	6	MM5000036726	CTGCGCTCTTTAAGGCGATTGG
Vimp-1	-1	2	MM5000028001	CTCAAAGCCCTCAGTCGAAGGG
Vimp-2	-1	2	MM5000028002	AGGATGTACCAGCCATAGCTGG
Wbp5-1	1	3	MM5000022657	GGAGGAGCTAGATGAGATCAGG
Wbp5-2	1	3	MM5000022658	CTTATAGTGACGCGTTGGAAGG
Wipi1-1	1	2	MM5000004257	GCCTTGGTTCCAATCGCTAGGG
Wipi1-2	1	2	MM5000004258	AGAGAAAACAGCTTGTAAACGG
Xbp1-1	-1	4	MM5000035983	TTCTGGGGAGGTGACAACTGGG
Xbp1-2	-1	4	MM5000035984	CAACAGTGTGACAGTCCATGGG
Yars1-1	-1	2	MM5000009767	GTACAAAGTAAGCCACGTGTGG
Yars1-2	-1	2	MM5000009768	ACGTGTGGCTTGCCCGTGGTGG
Yipf2-1	-1	2	MM5000022883	TGGTTGTGGTTTCAGGCATTGG
Yipf2-2	1	2	MM5000022884	ACAGCCACGTGCTCTCGGGAGG
Yipf5-1	1	3	MM5000016715	AGGATAGGCCTGAGTTGGCTGG
Yipf5-2	1	3	MM5000016716	TTGGAGGATAGGCCTGAGTTGG
Yipf6-1	-1	2	MM5000022897	TTGAGCACGAGCTCTGGAGGGG
Yipf6-2	-1	2	MM5000022898	TGTTCTTGAGCACGAGCTCTGG
Zbp1-1	-1	2	MM5000012743	GTCCTTTACCGCCTGAAGAAGG
Zbp1-2	-1	2	MM5000012744	TGCAGGTGTTGAGCGATGACGG
Zdhhc13-1	1	2	MM5000008415	GCTCCACGTTCCAGGATTTGG
Zdhhc13-2	1	2	MM5000008416	TTCCAGGATTTGGGCGACACGG
Zfp280b-1	1	2	MM5000014613	AGATCCAGAACCACAGCCGAGG
Zfp280b-2	1	2	MM5000014614	GCAGAGCTGATCTTCGTAGGGG
Zfp318-1	1	2	MM5000007875	TGTTAGCACACCAGAGCGGCGG
Zfp318-2	1	2	MM5000007876	TCTCCAGTGGATGGTCTGCAGG
Zfp831-1	1	3	MM5000019941	TTGAGGGGTTAGAGCCACGGGG
Zfp831-2	-1	3	MM5000019942	TTCTTCTTGGCAACCAGGGTGG
Zfyve21-1	-1	3	MM5000035911	TAAAACTCCGCCTCTCGGTGGG
Zfyve21-2	-1	3	MM5000035912	CGAAGCACATGCGCCGAAGTGG

2.2. Methods

2.2.1. Mice

C57BL/6, C57BL/6 x Ly5.1 F1 and B6-Cre-deleter mice were obtained from the Walter and Eliza Hall Institute (WEHI) breeding facility (Kew, Victoria). *Irf4*^{-/-} (Mittrücker et al., 1997), Rosa26-lox-STOP-lox-Cas9-IRES-GFP (Cas9^{LSL}) and CD23^{cre} mice have been described previously (Chu et al., 2016; Kwon et al., 2008). Cas9^{LSL} mice were bred with B6-Cre-deleter mice to generate the Constitutive Cas9 strain and were kindly provided by Marco Herold (WEHI, Parkville, Victoria). The *Irf4*-GFP reporter mice are unpublished and were kindly provided by Axel Kallies (Peter Doherty Institute, Parkville, Victoria). Experimental mice were bred and maintained on a C57BL/6 genetic background and housed in the WEHI animal facility in a specific pathogen free environment (Parkville, Victoria). Animal experiments were conducted in accordance with protocols approved by the WEHI animal ethics committee.

2.2.2. B cell isolation and culture

Naïve splenic B cells were isolated using a B Cell Isolation Kit (Miltenyi Biotech) and labelled with the division tracking dye, Cell Trace Yellow (CTY; Invitrogen). B cells were cultured in B cell medium (**Table 2.2**) stimulated with LPS (Sigma) at 10 µg/mL, mouse Interleukin-4 (IL-4; R&D) at 10 ng/mL, mouse Interleukin-5 (IL-5; R&D) at 5 ng/mL and mouse CD40 ligand (CD40L; R&D) at 50 ng/mL. All cultures were incubated at 37 °C with 5 % CO₂.

2.2.3. Flow cytometry and cell sorting

All monoclonal antibodies used in this work are listed in **Table 2.4**. All staining was performed in the presence of 2.4G2 (Produced in house) to block Fc receptors and prevent non-specific binding. Intracellular staining was performed using BD Cytofix/Cytoperm (BD Biosciences). Before analysing, samples were resuspended in FACS buffer (**Table 2.2**) containing 1 µg/mL propidium iodide (PI; Sigma-Aldrich), 0.5 µg/mL SYTOX Blue (ThermoFisher Scientific) or 1 µg/mL

FluoroGold (Sigma-Aldrich). For intracellular stained samples, 0.5 $\mu\text{L/mL}$ fixable viability dye eFluoro-506 (ThermoFisher Scientific) or 1 $\mu\text{L/mL}$ eFluoro-780 (eBioScience) was included in the extracellular staining cocktail. Absolute cell number was determined by adding 1×10^4 calibration beads (BD Biosciences) to each sample prior to analysis and calculating the ratio of beads to live cells. Samples were analysed using a BD FACSCantoII or BD LSRFortessa (BD Biosciences). Cell sorting was performed on a BD FACSARIAIII or BD FACSARIA Fusion (BD Biosciences) with the assistance of the WEHI Flow Cytometry facility.

2.2.4. Enzyme-linked immunosorbent assay (ELISA)

Supernatant was removed from B cell cultures and stored at -20°C until analysis. 96-well plates were coated with anti-IgM (1 $\mu\text{g/mL}$; Southern Biotech) overnight at room temperature (RT). Plates were washed with PBS/Tween20 (0.04 %), PBS then water. Culture supernatant or IgM standard (Sigma-Aldrich) diluted in ELISA block solution (**Table 2.2**) was added to the appropriate wells and incubated for 4 hours at RT. Plates were washed and incubated with anti-IgM-HRP (1 $\mu\text{g/mL}$; Southern Biotech) diluted in ELISA block solution for 4 hours at RT. Plates were washed and bound IgM was visualized using ABTS substrate solution (**Table 2.2**). Colour development was determined using a Kinetic Microplate Reader (Molecular Devices) using wavelengths 415-492 nm.

2.2.5. Enzyme-linked immunospot (ELISpot)

Multiscreen HA plates were coated with anti-IgM in 0.2 M carbonate buffer (**Table 2.2**) and incubated at RT for 4 hours. Plates were washed with PBS before cells in B cell media (**Table 2.2**) were added at the indicated cell density. Plates were then incubated at 37°C 10% CO_2 for 14-18 hours. After incubation, plates were washed with PBS/Tween20 (0.04%), PBS then water. 1 $\mu\text{g/mL}$ anti-IgM-HRP was diluted in ELISA block solution and added to wells. IgM secreting cells were visualized by the addition of ELISpot substrate solution (**Table 2.2**). Counting was performed using an AID EliSpot reader (Autoimmun Diagnostika GMBH) and AID EliSpot 7.0iSpot software (Autoimmun Diagnostika GMBH).

2.2.6. CRISPR gRNA libraries

Individual gRNAs were obtained from the Sanger Arrayed Mouse Whole Genome Lentiviral CRISPR Library (Sigma-Aldrich). This library consists of gRNAs within the vector U6-gRNA:hPGK-Puro-2A-tagBFP. Sequences for all gRNAs used in this thesis are listed in **Table 2.9**. Glycerol stocks for each individual gRNA were used to inoculate LB broth and were cultured overnight at 37 °C. Plasmid DNA was then purified using a QIAprep spin minikit (Qiagen) according to manufacturer's instructions and gRNAs were stored at -20 °C until use. The genome-wide mouse lentiviral gRNA library used in this thesis was developed by the Yusa laboratory (Koike-Yusa et al., 2014) and was generously provided to me by Marco Herold.

2.2.7. Production of lentiviral vectors

293T cells were maintained in DMEM supplemented with 10 % FCS. 16 hours prior to transfection, cells were plated in DMEM/10 % FCS at a density of 2×10^4 cells per well (96-well plate) or 1.5×10^6 cells per 10 cm² plate. pMD1-gag-pol, pCAG-Eco, pRSV-REV and gRNA plasmids were combined at a ratio of 3:2:2:3 in FCS-free DMEM at a total volume of 100 µL and incubated at RT for 10 minutes. For 96-well plate transfections and 10 cm² plate transfections the total DNA content was 2 µg and 5 µg respectively. Fugene6 Transfection Reagent (Promega) was added to the plasmid mix at a ratio of 6 µL Fugene6 to 1 µg DNA and mixes were incubated for a further 30 minutes at RT. The amount of the DNA-Fugene6 mixture added to 96-well plates and 10 cm² plates was 10 µL and 100 µL respectively. Lentivirus containing supernatant was collected after 48 hours, and either used immediately or frozen and stored at -80 °C until use.

2.2.8. Transduction of primary B cells

Non-tissue culture treated 96 well plates were coated with RetroNectin (32 µg/mL; produced in house) for a minimum of 4 hours at 4 °C. Plates were blocked with PBS/2 % BSA for 30 minutes at RT prior to the addition of cells and

lentivirus supernatant. Plates were then centrifuged at 1200 rpm for 90 minutes at 28 °C. Following centrifugation, cells were resuspended in B cell medium containing LPS±IL-4 and cultured for a further 3-4 days before analysis.

2.2.9. Irradiation and fetal liver reconstitutions

Cells from Ly5.1 (CD45.1⁺) and *Irf4*^{-/-} (CD45.2⁺) E14.5 foetal livers were combined at a ratio of 1:1 and transferred intravenously into lethally irradiated (2x550 rads) C57BL/6 x Ly5.1 F1 recipients (CD45.1⁺CD45.2⁺). Mice were left to reconstitute for 8 weeks before analysis.

2.2.10. RNA isolation

Cells were resuspended in RLT buffer (Qiagen,) with 1.6 mM β-mercaptoethanol and transferred to a QIA Shredder column (Qiagen) for cell lysis and homogenisation. RNA extraction was performed using RNeasy Plus Mini Kit (Qiagen) according to manufacturer's instructions. The extracted RNA was analysed on the Agilent4200 Tapestation and high-quality RNA with RIN values greater than 9 were used for library preparation.

2.2.11. RNA library preparation and sequencing analysis

Libraries were prepared from 100 ng of total RNA using the TruSeq RNA (Illumina) according to the manufacturer's instructions and sequenced on the NextSeq 500 (Illumina). The single-end 75 bp reads were demultiplexed using CASAVA v1.8.2 and Cutadapt (v1.9) was used for read trimming (Martin, 2011). The trimmed reads were mapped onto the mouse genome (mm10) using HISAT2 (Kim et al., 2019). *Featurecounts* from the *Rsubread* package (v1.34.7) with its in-built mm10 was used for read counting (Liao et al., 2013, 2019). Genes with a count per million (CPM) > 0 in at least 3 samples were included for further analysis. Count data were normalised using the trimmed mean of M-values (TMM) method and differential gene expression analysis was performed using the limma-voom pipeline (*limma* version 3.40.6) (Law et al., 2014). Heatmaps of

logCPM were generated using *pheatmap*. GSEA-2.2.2 was used for Gene set enrichment analysis (GSEA). Gene ontology (GO) analysis was performed using *Metascape*. The R/Bioconductor package *ggplot2* (version 3.2.1) was used to generate the volcano plots.

2.2.12. gDNA isolation

Sorted cells from multiple timepoints were frozen and stored at -80 °C so that all samples were processed at the same time. Cells were resuspended in PBS with Proteinase K and gDNA was purified using a DNeasy blood and tissue kit (Qiagen). DNA concentration was determined using a Qubit dsDNA high sensitivity assay kit (Invitrogen).

2.2.13. DNA library preparation and sequencing analysis

Next-generation sequencing libraries were prepared from 100 ng total gDNA following a protocol previously optimised by the WEHI genomics facility. In brief, samples were amplified using primer sequences obtained from Koike-Yusa et al. 2014, ligated to Illumina adapters and purified. Six technical library preparation replicates were performed for each sample. Libraries were pooled and sequenced on a MiSeq (Illumina) to a depth of 1-2 million reads per replicate for experimental samples and 5 million reads per replicate for the starting library samples. The Fastq files generated by sequencing were aligned to the Yusa mouse genome-wide gRNA library sequence file. Data analysis was performed using the MAGeCK-Flute pipeline (Li et al., 2015; Li et al., 2014; Wang et al., 2019). MAGeCK performs quality control analysis on sequencing files and calculates a beta score for each gene between samples to estimate gene effects.

Chapter 3. Identification of positive regulators of B cell differentiation, proliferation, and antibody secretion

3.1. Introduction

The differentiation of mature B cells into Antibody Secreting Cells (ASCs) is an essential part of the adaptive immune response. The antibodies that these cells produce are important for the elimination of infecting pathogens and the persistent secretion of these antibodies after pathogen clearance provides long-term protection against re-infection. Despite the importance of these cells, we still lack a complete understanding of the factors that drive B cell differentiation, ASC homing, long-term survival and the secretion of antibodies. Identifying the genes that are essential for ASC biology may allow more effective treatment of the plasma cell malignancy multiple myeloma and autoimmune diseases where antibodies directed against self-proteins are the drivers of immune pathology, including systemic lupus erythematosus.

The generation, survival and function of ASCs is regulated by a triad of transcription factors; Blimp-1, Irf4 and Xbp1 (Nutt et al., 2015). B lymphocyte induced maturation protein-1 (Blimp-1), encoded by *Prdm1*, is important for the terminal differentiation of both B and T lymphocytes (Kallies and Nutt, 2007). While Blimp-1 is not required for the initiation of the B cell differentiation process, it is essential for the formation of ASCs, both *in vitro* and *in vivo* (Kallies et al., 2007; Shapiro-Shelef et al., 2003). A major function of Blimp-1 during the terminal differentiation process is to silence the expression of genes responsible for maintaining B cell identity (Lin et al., 2002; Lin et al., 1997; Minnich et al., 2016; Shaffer et al., 2002). In ASCs, Blimp-1 is required to maintain the expression of genes involved antibody secretion, either through direct activation or through the recruitment of chromatin modifying complexes (Minnich et al., 2016; Tellier et al., 2016). Irf4 is an essential regulator of many processes throughout B cell development and antigen response (De Silva et al., 2012). Irf4 functions in a dose-dependent manner, where lower expression in activated B cells is required for class-switch recombination and the formation of germinal centres (GCs), while high expression of Irf4 drives ASC differentiation (Klein et al., 2006; Ochiai et al., 2013; Sciammas et al., 2006; Willis et al., 2014). It is also essential for the survival of mature ASCs as the deletion of *Irf4* in plasma cells *in vivo* results in the rapid loss of the ASC compartment (Tellier et al., 2016). The major function of ASCs is antibody secretion; therefore, they perform a massive amount of protein

synthesis, post-translational modifications and exporting of proteins from the cell. This makes them extremely sensitive to endoplasmic reticulum (ER) stress and, as a consequence, they are particularly dependent on ER stress responses including the unfolded protein response (UPR) (Gass et al., 2002). X-box-binding protein-1 (Xbp1) is a regulator of the UPR and is upregulated in protein-secreting cells, including ASCs (Iwakoshi et al., 2003). Differentiation into ASCs is possible in the absence of Xbp1, however, the ER morphology of Xbp1 deficient ASCs is altered and the antibody secreting capacity of these cells is severely reduced (Taubenheim et al., 2012; Tellier et al., 2016; Todd et al., 2009). It is highly likely that there are additional genes which are essential for the generation, maintenance and function of ASCs, therefore I sought to perform a screen to identify novel regulators of ASC biology.

The differentiation process requires the repression of genes that determine B cell identity and the expression of a collection of genes that are responsible for ASC biology (Nutt et al., 2011). A previous study examined the precise alterations in gene expression that occurs during the process of B cell terminal differentiation and defined the ASC gene signature (Shi et al., 2015). Transcriptional analysis was performed on B cell and ASC subsets and the transcriptomes were compared to identify the genes whose expression was increased in ASC populations compared to B cell subsets (> 3-fold increased expression in ASC subsets). This list, named the ASC gene signature, consists of 301 genes whose expression is upregulated as naïve follicular B cells differentiate into ASCs. In addition to known regulators of ASC biology, including *Xbp1*, *Irf4*, *Prdm1*/Blimp-1, this signature contained many genes whose functions have either not been characterized or have not been examined in the context of ASCs. To interrogate their function, I developed a CRISPR-Cas9 mediated arrayed targeted screen in primary B cells, with the ability to measure multiple parameters in parallel, including antibody secretion. I have applied this system to the ASC gene signature to identify genes essential for differentiation, survival, proliferation and secretion capacity of ASCs. I have combined this screening with more focused investigation of selected candidates to more finely dissect the cellular processes that these genes regulate. Through this process I have identified

previously unstudied genes that regulate ASC biology that therefore present new avenues for targeting ASCs for the treatment of ASC-based pathologies.

3.2. Results

3.2.1. Development of an arrayed targeted CRISPR screen for primary B cells
I sought to establish a CRISPR-Cas9 based screening system which would allow the identification of genes that are essential for the generation, survival and antibody secreting capacity of ASCs. While conventional pooled CRISPR-Cas9 screens are capable of detecting genes required for differentiation and survival, they are not able to assess defects in antibody secretion. Therefore, I designed a system to perform a targeted screen on primary B cells, using an arrayed CRISPR gRNA library, which would be suitable to identify genes essential for differentiation, survival and antibody secretion (**Figure 3.1**).

I first optimized the transfection of 293T cells with gRNAs in a 96-well format. Using the manufacturers recommended cell density, DNA content, ratio of Fugene6 to DNA and incubation time (1×10^4 cells, 5 μ g total DNA, 30 μ L Fugene6, 20 minute incubation), the initial transfection efficiency was approximately 30 % (**Figure 3.2a**). After several rounds of optimisation (2×10^4 cells, 2 μ g total DNA, 12 μ L Fugene6, 5 minute incubation of DNA followed by 30 minute incubation with DNA and Fugene6), the transfection rate was close to 100 % (**Figure 3.2b**). The 96-well transduction efficiency of primary mouse B cells was similarly improved from a starting efficiency of approximately 30 % (B cells activated for 24 hours with LPS, spin infection with 4 μ g polybrene, 2400 rpm for 90 minutes), to a method where transduction rates were above 80 % (B cells activated for 24 hours with LPS, spin infection with RetroNectin coated plates, 1200 rpm for 90 minutes) (**Figure 3.2c,d**). Importantly, the use of previously prepared and frozen lentiviral supernatant did not result in a decrease in transduction efficiency, indicating that the lentiviral gRNA library could be produced in advance and stored until use (**Figure 3.2d**).

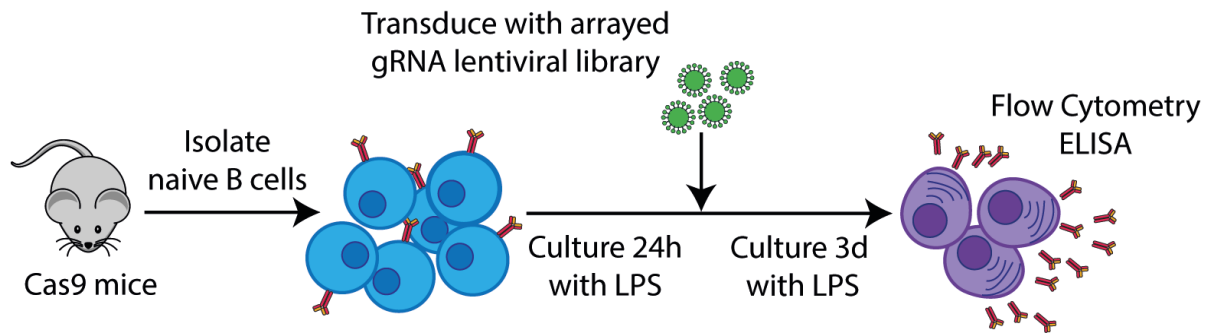


Figure 3.1 Overview of experimental workflow for targeted CRISPR/Cas9 screen

Naïve splenic B cells were isolated from Cas9 expressing transgenic mice, activated with LPS and transduced with an arrayed lentiviral library co-expressing specific gRNAs and BFP. Three days after transduction, cells were analysed by flow cytometry and culture supernatant was analysed by ELISA to determine antibody production.

As a proof of concept, I first targeted two genes encoding cell surface proteins, *Sdc1* encoding CD138 and *Slc3a2* encoding the CD98 heavy chain. Naïve splenic B cells were isolated from transgenic mice constitutively expressing Cas9 and stimulated for 24 hours with LPS before transduction with gRNAs targeting either *Sdc1* or *Slc3a2*. At 3 days post-transduction, cells transduced with gRNAs targeting *Sdc1* had a 4-fold decrease in the proportion expressing CD138 (**Figure 3.3a**). Similarly, cells transduced with *Slc3a2* gRNAs displayed a reduction in the mean fluorescence intensity (MFI) of surface CD98 (**Figure 3.3b**).

To test that this system was also capable of blocking the differentiation of B cells, I next targeted genes known to be essential for the differentiation process, *Irf4* and *Prdm1* (Mittrücker et al., 1997; Shapiro-Shelef et al., 2003). At 3 days post-transduction, cells transduced with gRNAs targeting *Irf4* or *Prdm1* showed an 80-90 % decrease in the proportion of differentiated cells as determined by the expression of CD138 (**Figure 3.3c**). This inhibition of differentiation was specifically due to the targeting of either *Irf4* or *Prdm1*, as cells that were transduced with gRNAs directed against *Plpp5*, a gene that does not influence B cell differentiation (Trezise et al., 2018), did not show this effect. To examine the antibody secreting capacity of the transduced cells, the concentration of IgM in the culture supernatant was measured. Cells transduced with gRNAs targeting either *Irf4* or *Prdm1* showed a reduction of at least 70 % in IgM secretion relative to the control while cells transduced with *Plpp5* targeting gRNAs did not display a decrease in antibody secretion (**Figure 3.3d**). From this, I conclude that, despite the short timeframe, this system is capable of detecting genes that are essential for B cell differentiation and antibody secretion.

These optimization experiments were performed using the T cell independent LPS as the stimulation condition, however, it is desirable to be able to perform this screen under multiple conditions. To determine whether this screening system could be adapted to identify genes important for differentiation in response to conditions that mimic T-dependent stimulation, naïve WT and Cas9 B cells were activated with CD40L/IL-4/IL-5. Although WT and Cas9 B cells had no difference in differentiation to LPS, Cas9 B cells showed reduced differentiation following CD40L/IL-4/IL-5 stimulation (**Figure 3.4**). This impaired differentiation response means that it is not suitable to perform this screen under CD40L/IL-4/IL5 stimulation, therefore, all further experiments were conducted using LPS.

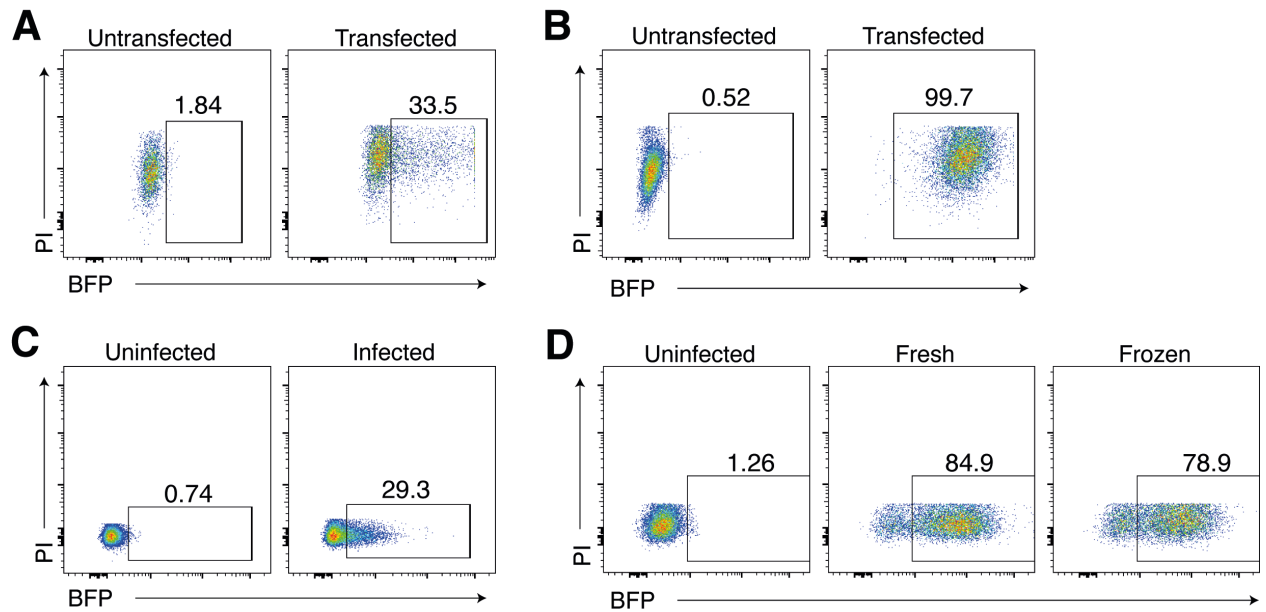


Figure 3.2 Optimization of a 96-well plate transfection and primary mouse B cell transduction method

(A) Efficiency of transfection of 293T cells in 96-well plates as measured by the proportion of BFP⁺ cells at 48 hours post-transfection, prior to protocol optimization. (B) Efficiency of transfection of 293T cells in 96-well plates as measured by the proportion of BFP⁺ cells at 48 hours post-transfection, using optimised transfection protocol. (C) Transduction of primary mouse B cells using 4 μ g/mL polybrene. (D) Transduction of primary mouse B cells using RetroNectin coated plates and either freshly produced or frozen and thawed lentivirus supernatant. Boxes show the proportion of live (propidium iodide (PI)⁻) BFP⁺ cells.

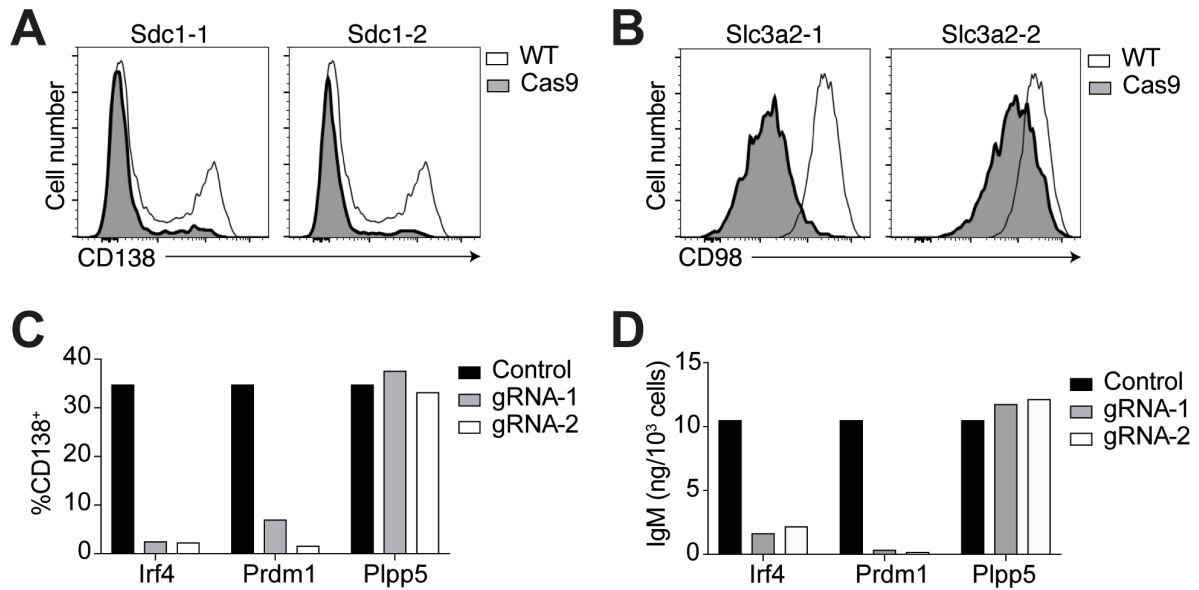


Figure 3.3 Effects of gRNAs can be observed at 3 days post-transduction
 (A-B) Naïve splenic B cells from WT or Cas9 transgenic mice were activated with LPS and transduced with independent gRNAs targeting (A) *Sdc1* or (B) *Slc3a2*. At 3 days post-transduction, the surface expression of CD138 and CD98 was assessed by flow cytometry. (C) Naïve splenic B cells from Cas9 transgenic mice were activated with LPS and transduced with gRNAs targeting *Irf4*, *Prdm1* or *Plpp5*. At 3 days post-transduction, the proportion of CD138⁺ cells was assessed by flow cytometry. (D) Concentration of IgM in the culture supernatants from C as determined by ELISA.

I next generated an arrayed gRNA lentivirus library using the optimised protocol. Transfections were performed in batches of 36 or 72 gRNAs in parallel and at 48 hours post-transfection lentivirus containing supernatant was transferred to fresh 96-well plates and stored at -80 °C until use. Every library plate generated contained at least 2 gRNAs targeting *Irf4*, which served as a positive control for a block in differentiation, and 2 empty wells that served as uninfected controls. All transfection efficiencies were checked by flow cytometry and any gRNA with a transfection efficiency of less than 80 % was remade.

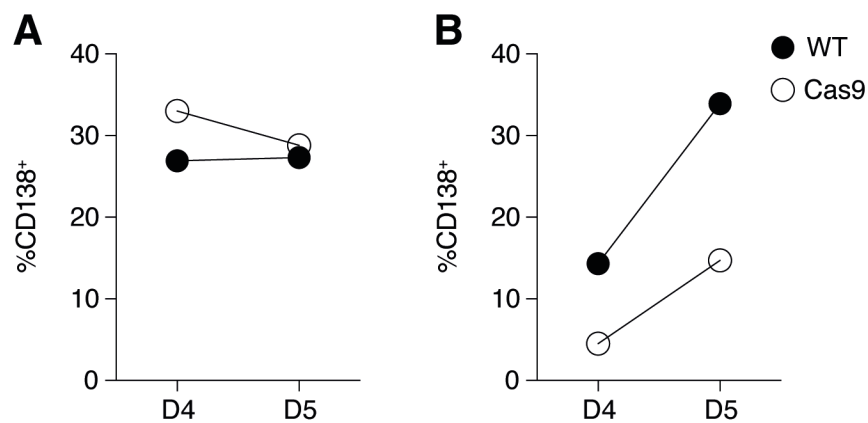


Figure 3.4 Cas9 B cells have impaired differentiation in response to CD40L/IL-4/IL-5 stimulation

Naïve splenic B cells from Cas9 transgenic mice were activated with (A) LPS or (B) CD40L/IL-4/IL-5. At the indicated timepoints post stimulation the proportion of CD138⁺ cells was assessed by flow cytometry. Representative of 2 independent experiments.

3.2.2. A targeted CRISPR screen reveals the essential regulators of ASC differentiation

After demonstrating that this system was capable of detecting blocks in B cell differentiation, I used it to interrogate the ASC gene signature to identify novel genes that were essential for the generation of ASCs. Naïve splenic B cells were isolated from Cas9 transgenic mice and stimulated for 24 hours with LPS before transduction with an arrayed lentiviral library containing 2 gRNAs against each protein-coding gene within the ASC gene signature (**Table 3.1**). Following transduction, the cells were returned to culture under LPS stimulation for a further 3 days before analysis. The effect of each gRNA on differentiation was determined by the proportion of BFP⁺ cells that expressed CD138. The cut-off for genes of interest was arbitrarily set as having a reduction of at least 50 % in the proportion of differentiated cells, relative to the uninfected control on each plate (**Figure 3.5**). As was expected, and in agreement with the validation experiments, gRNAs targeting *Irf4*, *Prdm1* or *Sdc1* all resulted in a decrease in CD138⁺ cells. In addition to this, I also observed a reduction in differentiated cells following transduction with gRNAs targeting *Hspa5*, which encodes Grp78/BiP, and *Sec61a1*. There was good agreement between the effect of gRNA pairs directed against the same gene, and a consistent effect of targeting the same gene across replicate screens (**Figure 3.6**).

To determine whether targeting these genes was causing a complete block or in differentiation or a delay in the onset of differentiation that would not be detected the single timepoint examined in the screen, I repeated the assay, focusing on genes of interest and examining differentiation at multiple timepoints. *Irf4* and *Prdm1* are known to be essential for the differentiation of B cells into ASCs and, as expected, I detected fewer CD138⁺ cells that had been transduced with gRNAs targeting either of these genes at any examined timepoint (**Figure 3.7**). Similarly, there was a consistent reduction in the proportion of CD138⁺ cells observed following transduction with gRNAs targeting *Sec61a1* or *Hspa5* at all time points. In agreement with the screen optimization experiments, targeting *Plpp5* did not have any effect on the rate of differentiation.

Table 3.1 Genes included in ASC gene signature targeted gRNA library

Gene name	MGI ID
1700017B05Rik	MGI:1921461
4632428N05Rik	MGI:1921298
Abcb1b	MGI:97568
Ada	MGI:87916
Adk	MGI:87930
Alad	MGI:96853
Alcam	MGI:1313266
Aldh18a1	MGI:1888908
Aldh9a1	MGI:1861622
Alg2	MGI:1914731
Ap2m1	MGI:1298405
Arf4	MGI:99433
Arfgap3	MGI:1913501
Asns	MGI:1350929
Atat1	MGI:1913869
Atf5	MGI:2141857
Atl2	MGI:1929492
Atp6v0a1	MGI:103286
BC003331	MGI:2385108
Bckdk	MGI:1276121
Bet1	MGI:1343104
Bhlha15	MGI:891976
Bst2	MGI:1916800
Btd	MGI:1347001
Cacna1h	MGI:1928842
Calr	MGI:88252
Capn5	MGI:1100859
Cars	MGI:1351477
Ccdc117	MGI:2144383
Ccpq1	MGI:1196419

Gene name	MGI ID
Cd28	MGI:88327
Cdv3	MGI:2448759
Chid1	MGI:1915288
Chpf	MGI:106576
Chst1	MGI:1924219
Cited2	MGI:1306784
Clptm1l	MGI:2442892
Cltb	MGI:1921575
Cnpy2	MGI:1928477
Cnst	MGI:2445141
Cope	MGI:1891702
Coro2b	MGI:2444283
Cpeb2	MGI:2442640
Cpox	MGI:104841
Creb3l2	MGI:2442695
Creg1	MGI:1344382
Creld2	MGI:1923987
Ctse	MGI:107361
D17Wsu104e	MGI:2156020
Dap	MGI:1918190
Ddost	MGI:1194508
Dennd5b	MGI:2444273
Derl1	MGI:1915069
Derl3	MGI:1917627
Desi1	MGI:106313
Dhdds	MGI:1914672
Dnajb11	MGI:1915088
Dnajb9	MGI:1351618
Dnajc3	MGI:107373
Dpagt1	MGI:1196396

Gene name	MGI ID
Edem1	MGI:2180139
Edem2	MGI:1915540
Edem3	MGI:1914217
Eli2	MGI:2183438
Endou	MGI:97746
Enpp1	MGI:97370
Entpd1	MGI:102805
Epcam	MGI:106653
Ergic1	MGI:1914708
Erlec1	MGI:1914003
Erp44	MGI:1923549
Fam214a	MGI:2387648
Fam46c	MGI:1921895
Fcer1g	MGI:95496
Fkbp11	MGI:1913370
Fkbp2	MGI:95542
Fndc3a	MGI:1196463
Fndc3b	MGI:1919257
Fos	MGI:95574
Fut1	MGI:109375
Fut8	MGI:1858901
Galnt2	MGI:894694
Gfpt1	MGI:95698
Glb1	MGI:88151
Glpr1	MGI:1920940
Glt8d1	MGI:1923735
Gne	MGI:1354951
Gpr155	MGI:1915776
Gpr55	MGI:2685064
Gvin1	MGI:1921808
H13	MGI:95886

Gene name	MGI ID
H1f0	MGI:95893
Hdlbp	MGI:99256
Herpud1	MGI:1927406
Hid1	MGI:2445087
Hist1h1c	MGI:1931526
Hsd11b1	MGI:103562
Hsp90b1	MGI:98817
Hspa13	MGI:1309463
Hspa5	MGI:95835
Htatif2	MGI:1859271
Hyou1	MGI:108030
Idh2	MGI:96414
Ift20	MGI:1915585
Iqcb1	MGI:2443764
Irf4	MGI:1096873
Isg20	MGI:1928895
Itm2c	MGI:1927594
Kcnn4	MGI:1277957
Kdelr1	MGI:1915387
Kdelr2	MGI:1914163
Krtcap2	MGI:1913309
Larp1b	MGI:1914604
Leprotil1	MGI:1915442
Lman1	MGI:1917611
Lrrc59	MGI:2138133
Ly6c1	MGI:96882
Ly6c2	MGI:3712069
Ly6k	MGI:1923736
Maged1	MGI:1930187
Magt1	MGI:1914325
Man1b1	MGI:2684954

Gene name	MGI ID
Manea	MGI:2444484
Mars	MGI:1345633
Mcfcd2	MGI:2183439
Mettl9	MGI:1914862
Mgat2	MGI:2384966
Mnda	MGI:3041120
Mrp63	MGI:1915090
Mt1	MGI:97171
Mt2	MGI:97172
Mtdh	MGI:1914404
Mthfd2	MGI:1338850
Mzb1	MGI:1917066
Nans	MGI:2149820
Nars	MGI:1917473
Ndufa1	MGI:1929511
Neil1	MGI:1920024
Nucb1	MGI:97388
Nudt22	MGI:1915573
Oosp1	MGI:2149290
Os9	MGI:1924301
Oxct1	MGI:1914291
P4hb	MGI:97464
Pck2	MGI:1860456
Pdia6	MGI:1919103
Pon2	MGI:106687
Pon3	MGI:106686
Ppapdc1b	MGI:1919160
Pqlc3	MGI:2444067
Praf2	MGI:1859607
Prdm1	MGI:99655
Prdx4	MGI:1859815

Gene name	MGI ID
Prg2	MGI:103294
Prmt7	MGI:2384879
Prrc1	MGI:1916106
Pycr1	MGI:2384795
Qpctl	MGI:1914619
Rapgef3	MGI:2441741
Reln	MGI:103022
Rexo2	MGI:1888981
Rgcc	MGI:1913464
Rhob	MGI:107949
Rhobtb1	MGI:1916538
Rpl10	MGI:105943
Rpl15	MGI:1913730
Rpl23a	MGI:3040672
Rpn1	MGI:98084
Rpn2	MGI:98085
Rps27l	MGI:1915191
Rps6	MGI:98159
Sdc1	MGI:1349162
Sdf2l1	MGI:2149842
Sec11c	MGI:1913536
Sec22b	MGI:1338759
Sec24a	MGI:1924621
Sec24d	MGI:1916858
Sec61a1	MGI:1858417
Sec63	MGI:2155302
Sel1l	MGI:1329016
Selk	MGI:1931466
Selm	MGI:2149786
Sepp1	MGI:894288
Serinc5	MGI:2444223

Gene name	MGI ID
Serpina3f	MGI:2182838
Sil1	MGI:1932040
Slamf7	MGI:1922595
Slc12a4	MGI:1309465
Slc33a1	MGI:1332247
Slc35b1	MGI:1343133
Slc39a11	MGI:1917056
Slc39a4	MGI:1919277
Slc39a7	MGI:95909
Slc3a2	MGI:96955
Slc44a1	MGI:2140592
Slc7a5	MGI:1298205
Slpi	MGI:109297
Snd1	MGI:1929266
Snx9	MGI:1913866
Spcs1	MGI:1916269
Spcs2	MGI:1913874
Spcs3	MGI:1923937
Spint2	MGI:1338031
Spon1	MGI:2385287
Srp54a	MGI:1346087
Srp54c	MGI:3714359
Srpr	MGI:1914648
Srprb	MGI:102964
Ssr1	MGI:105082
Ssr2	MGI:1913506
Ssr3	MGI:1914687
Ssr4	MGI:1099464
St6gal1	MGI:108470
Stt3a	MGI:105124
Sumo2	MGI:2158813

Gene name	MGI ID
Surf4	MGI:98445
Tapbpl	MGI:2384853
Tigit	MGI:3642260
Tmed2	MGI:1929269
Tmed3	MGI:1913361
Tmed9	MGI:1914761
Tmem176a	MGI:1913308
Tmem176b	MGI:1916348
Tmem184b	MGI:2445179
Tmem214	MGI:1916046
Tmem116	MGI:1924712
Tmem248	MGI:1918917
Tmem258	MGI:1916288
Tmem39a	MGI:1915096
Tmem66	MGI:1915137
Tnfrsf17	MGI:1343050
Tns3	MGI:2443012
Top1	MGI:98788
Tpst1	MGI:1298231
Trabd	MGI:1915226
Tram2	MGI:1924817
Trp53inp1	MGI:1926609
Tvp23b	MGI:1914760
Txndc11	MGI:1923620
Txndc5	MGI:2145316
Uba5	MGI:1913913
Ubb	MGI:98888
Ube2j1	MGI:1926245
Ubxn4	MGI:1915062
Ufc1	MGI:1913405
Uso1	MGI:1929095

Gene name	MGI ID
Vcp	MGI:99919
Vimp	MGI:95994
Wbp5	MGI:109567
Wipi1	MGI:1261864
Xbp1	MGI:98970
Yars	MGI:2147627

Gene name	MGI ID
Yipf2	MGI:1922016
Yipf5	MGI:1914430
Yipf6	MGI:1925179
Zbp1	MGI:1927449
Zfp280b	MGI:1927865
Zfyve21	MGI:1915770

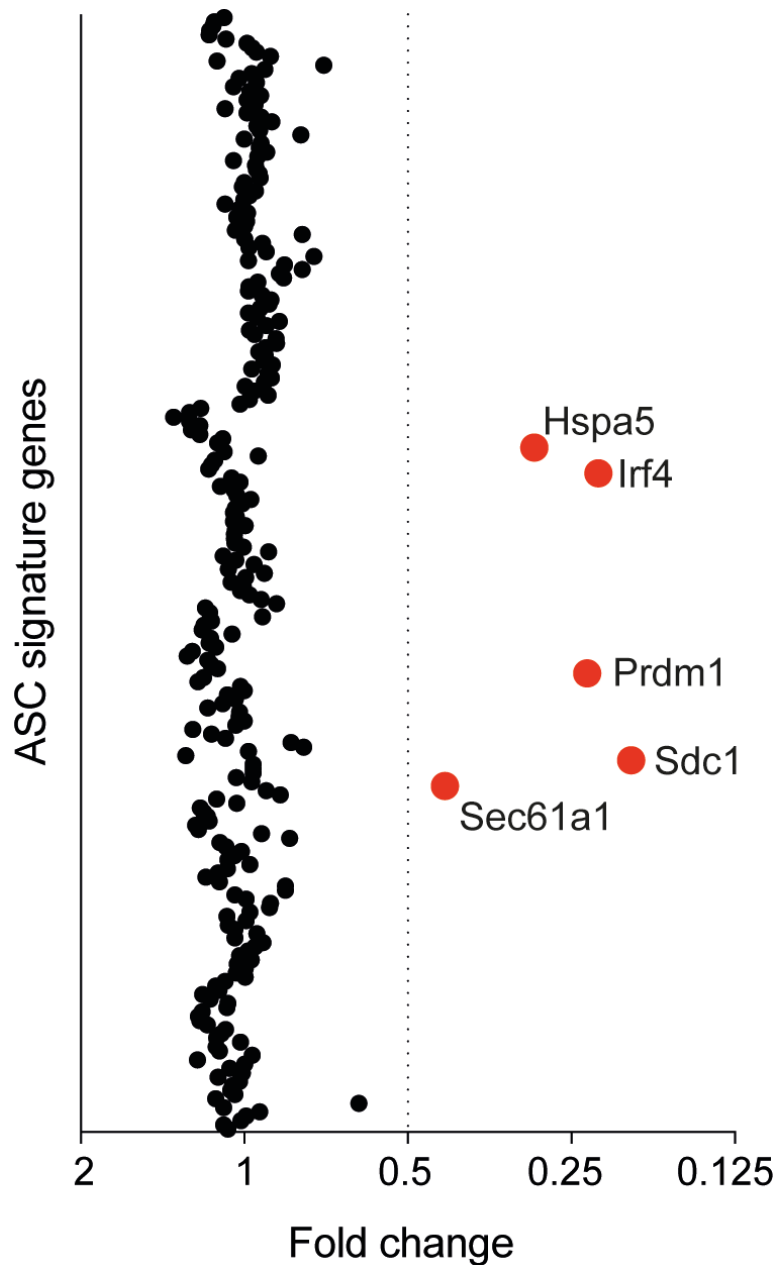


Figure 3.5 Identification of positive regulators of B cell differentiation

Experimental workflow is described in **Figure 3.1**. Each data point represents the average fold change in the proportion of CD138⁺ cells for both gRNAs targeted a particular gene relative to the untransduced controls on the same assay plate. Genes with a fold change of 0.5 or lower are labelled and highlighted in red. Data points represent the mean of 2 independent gRNAs from 2 replicate experiments.

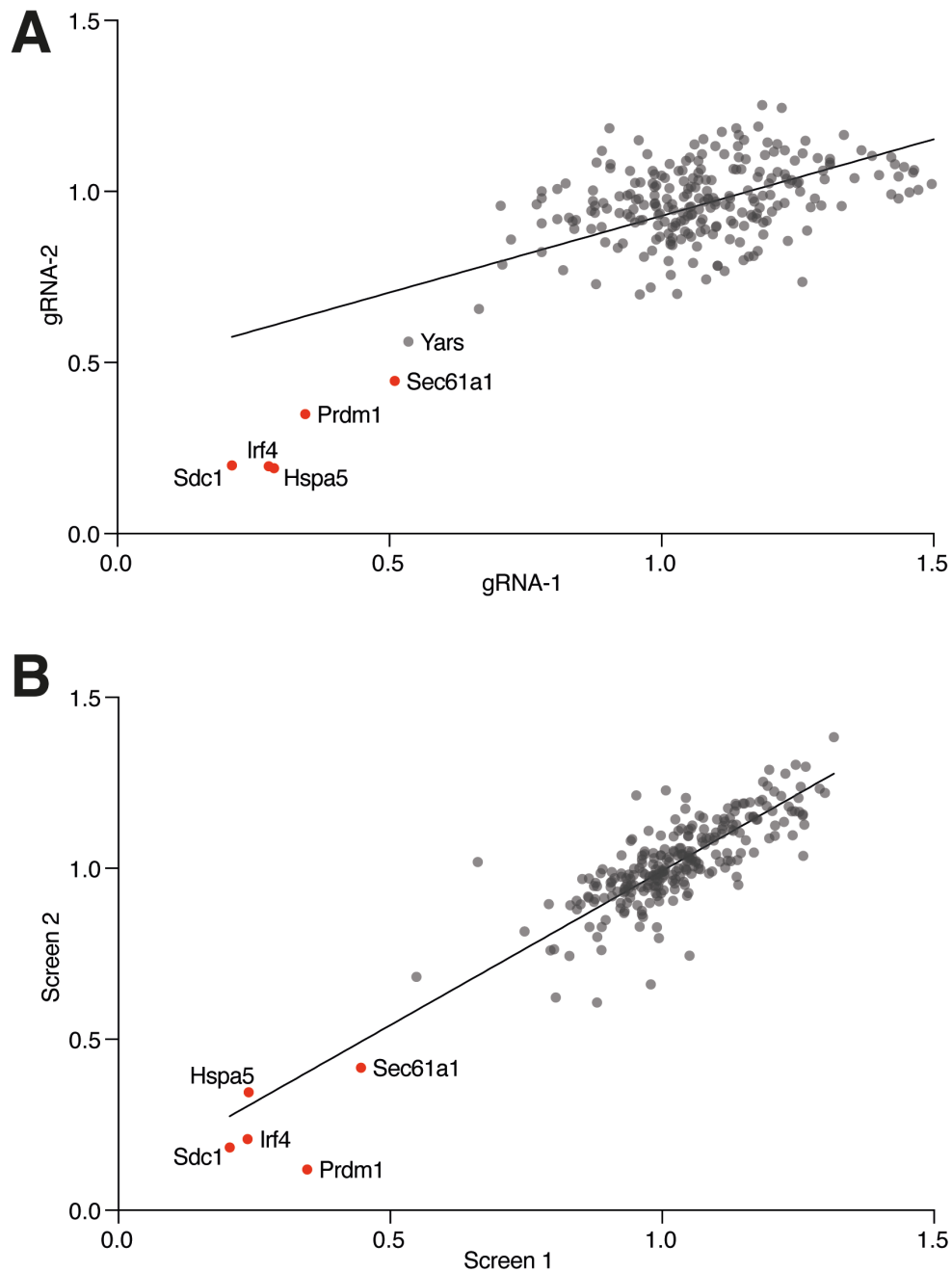


Figure 3.6 Correlation between effects of gRNAs within and between screens
*Experimental workflow as described in **Figure 3.1**. (A) Fold change in the proportion of CD138⁺ cells for each gRNA directed against the same gene in screen 1. (B) Average fold change in the proportion of CD138⁺ cells for each gene in replicate screens. Genes with a fold change of 0.5 or lower in both screens are labelled and highlighted in red.*

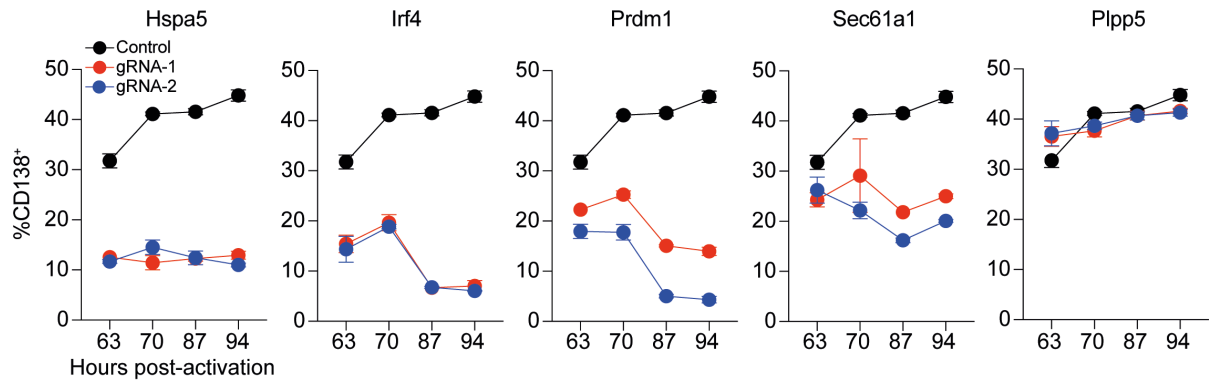


Figure 3.7 Differentiation of screen hits at multiple timepoints

Naïve Cas9 B cells were activated with LPS for 24 hours and transduced with gRNAs targeting the indicated genes. At the indicated time post-activation, the proportion of CD138⁺ cells was determined by flow cytometry. The untransduced control is shared between each plot. Points represent the mean of triplicates $\pm$ standard error. Representative of 2 independent experiments.

3.2.3. A targeted CRISPR screen reveals regulators of B cell survival and proliferation

This arrayed screening method is also suitable for identifying genes that influence cell number, either through affecting proliferation or cell survival. By examining the total live cell count instead of the proportion of CD138⁺ cells, I examined genes which caused a reduction in the total live cell number. As with the analysis of differentiation regulators, the cut-off for genes of interest was set as having an at least 50 % reduction in the total number of live cells, relative to the uninfected control for each plate. Through this method I identified 10 genes (*Cdv3*, *Hspa5*, *Irf4*, *Rpl10*, *Rpl15*, *Rpl23a*, *Rps6*, *Selk*, *Sumo2* and *Vcp*) which influenced cell survival and/or proliferation following activation with LPS (**Figure 3.8a**). *Rpl10*, *Rpl15*, *Rpl23a* and *Rps6*, encode ribosomal proteins. *Vcp* encodes p97, a major regulator of the ER associated degradation pathway (ERAD), which extracts misfolded proteins from the ER membrane and targets them for proteasomal degradation (Jarosch et al., 2002; Rabinovich et al., 2002; Ye et al., 2001). In addition to this, it has been implicated in the regulation of many cellular processes including autophagy, mitochondria associated degradation, ribosome quality control, transcription, DNA replication, the DNA damage response and endosomal trafficking (Hänzelmann et al., 2019; Xia et al., 2016). Given the roles of *Rpl10*, *Rpl15*, *Rpl23a*, *Rps6* and *Vcp* in essential cellular processes and their ubiquitous expression, I chose not to investigate these genes further. *Selk* encodes another component of the ERAD pathway and its interaction with p97 is essential for misfolded protein degradation (Du et al., 2010; Lee et al., 2015; Shchedrina et al., 2011). Additionally, *Selk* has been shown to regulate calcium flux in multiple immune cell types in response to a range of stimulation conditions and has been implicated in lymphocyte proliferation (Verma et al., 2011). *Sumo2* encodes a small ubiquitin-like molecule which can be attached to proteins involved in a range of cellular processes and modulate their activity, and has been implicated in the regulation of cell cycle (Eifler and Vertegaal, 2015; Wang et al., 2014). Interestingly, *Irf4* and *Hspa5* appear to influence both B cell differentiation and total live cell number. The remaining gene, *Cdv3*, does not have an ascribed function.

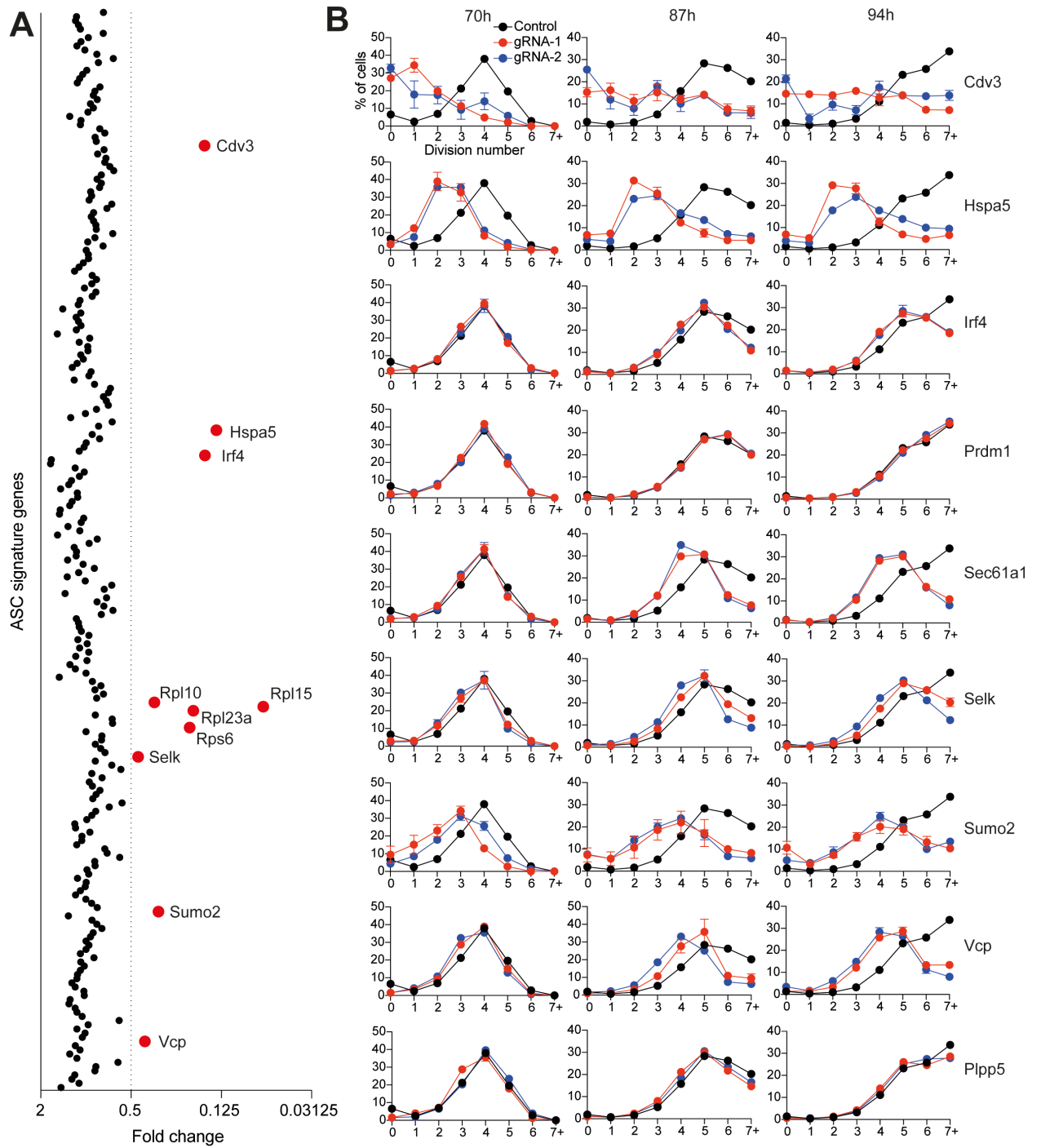


Figure 3.8 Identification of positive regulators of B cell proliferation

(A) *Experimental workflow is described in **Figure 3.1**. Each data point represents the average fold change in the number of live cells for both gRNAs targeting a particular gene relative to the untransduced controls on the same assay plate. Genes with a fold change of 0.5 or lower are labelled and highlighted in red. Data points represent the mean of 2 independent gRNAs from 2 replicate experiments.*

(B) *Naïve B cells from Cas9 transgenic mice were labelled with the division tracking dye Cell Trace Yellow (CTY), activated with LPS and transduced with gRNAs targeting the indicated genes. At the indicated timepoints, the dilution of CTY was assessed by flow cytometry.*

By using this screening method, I was unable to differentiate between genes that affected cell survival and genes that affected proliferation. To study these effects in more detail I used the same workflow as the screen but pre-labelled cells with cell trace yellow (CTY), a division tracking dye, which allowed the proliferative rate of the transduced cells to be examined at multiple timepoints. To ensure that the transduction with gRNAs was not affecting proliferation, *Prdm1* and *Plpp5*, which are not expected to influence cell division, were included. As expected, the CTY dilution profiles of cells transduced with gRNAs targeting *Prdm1* and *Plpp5* overlapped with the uninfected controls (**Figure 3.8b**). It has previously been demonstrated that *Irf4* deficient B cells have a reduced proliferative capacity in response to LPS when compared to WT B cells (Mittrücker et al., 1997). This defect was confirmed and was most notable at later timepoints. Targeting *Cdv3*, *Hspa5*, *Sumo2* or *Vcp* caused a dramatic reduction in proliferation capacity while targeting *Selk* resulted in a less severe alteration in cell division.

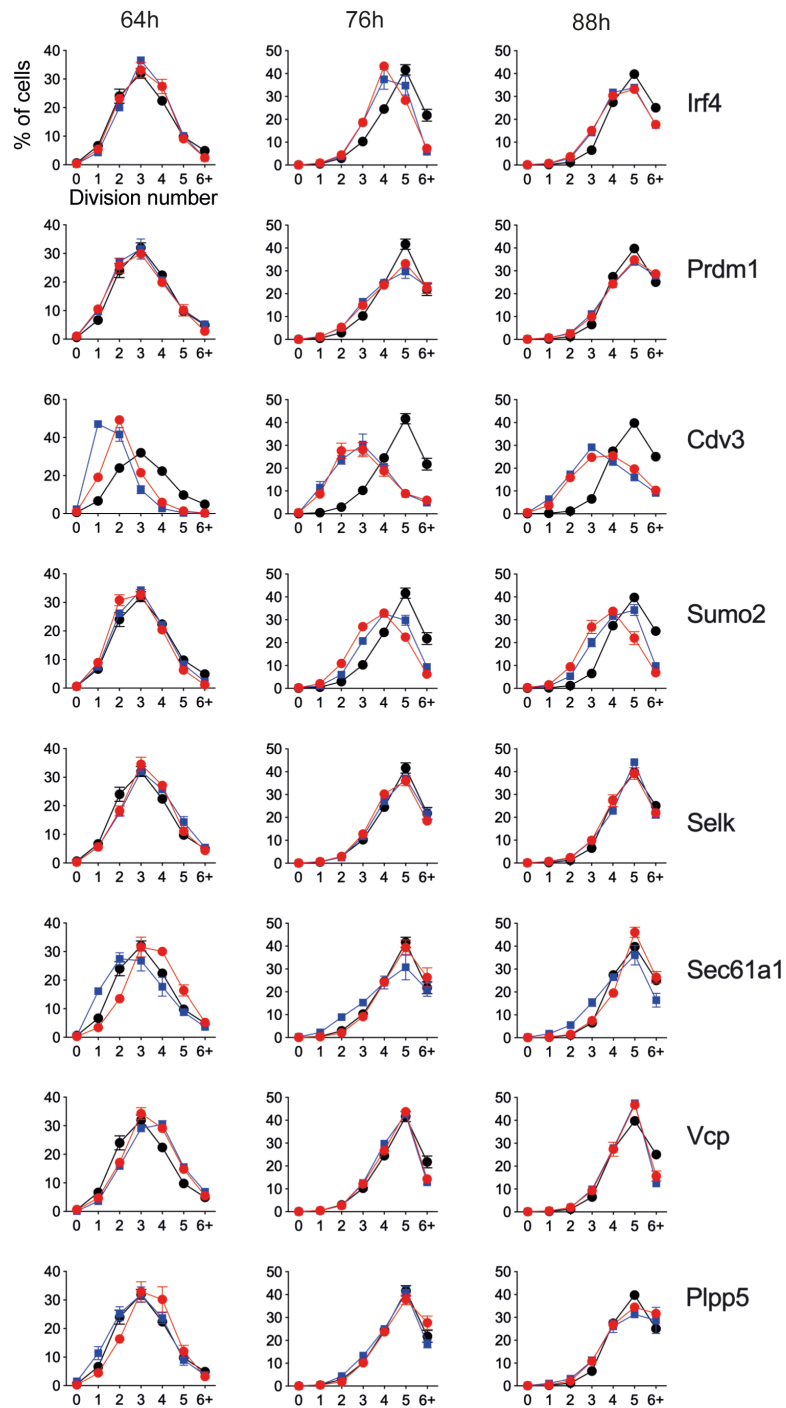


Figure 3.9 Influence of screen hits on B cell proliferation following CD40L/IL-4/IL-5 stimulation

Naïve B cells from Cas9 transgenic mice were labelled with CTY, activated with CD40L/IL-4/IL-5 and transduced with gRNAs targeting the indicated genes. At the indicated timepoints, the dilution of CTY was assessed by flow cytometry.

To determine if these effects on cell division were specific to the LPS response, I repeated the CTY assay on B cells activated with CD40L/IL-4/IL-5. While Cas9 B cells had impaired differentiation to CD40L/IL-4/IL-5, they were still able to proliferate following activation. In agreement with the LPS assay, targeting *Prdm1* and *Plpp5* had no effect on cell division, while targeting *Cdv3* or *Sumo2* resulted in a clear defect in proliferation (**Figure 3.9**). Surprisingly, targeting *Irf4*, *Selk*, or *Vcp* appeared to have no clear influence on cell division following CD40L/IL-4/IL-5 stimulation. This suggests that *Irf4*, *Selk* and *Vcp* may have a stimulation dependent role in regulating B cell proliferation, while *Cdv3* and *Sumo2* may influence cell division under a range of conditions.

3.2.4. A targeted CRISPR screen reveals regulators of LPS driven antibody secretion

The principal advantage of performing an arrayed CRISPR-Cas9 screen over a standard pooled screen is that it allows for the identification of genes that effect secretion. As the secretion of antibody is the predominant function of ASCs, I was interested in identifying which genes are essential for this process. Supernatant was isolated from the transduced cell cultures and the concentration of secreted IgM was determined by ELISA. To account for variation in cell numbers, results were normalized to IgM secretion per cell. Again, the cut-off for candidate regulators was set as having a 50 % reduction in IgM secretion per cell, relative to the uninfected plate control. Within the ASC gene signature there were 35 genes that influenced antibody secreting capacity (**Figure 3.10a**). The reduction in IgM secretion after targeting *Irf4*, *Prdm1* and *Sec61a1* is reflective of the block in differentiation (**Figure 3.5**), whereas the remaining 32 genes are potential regulators of antibody secretion.

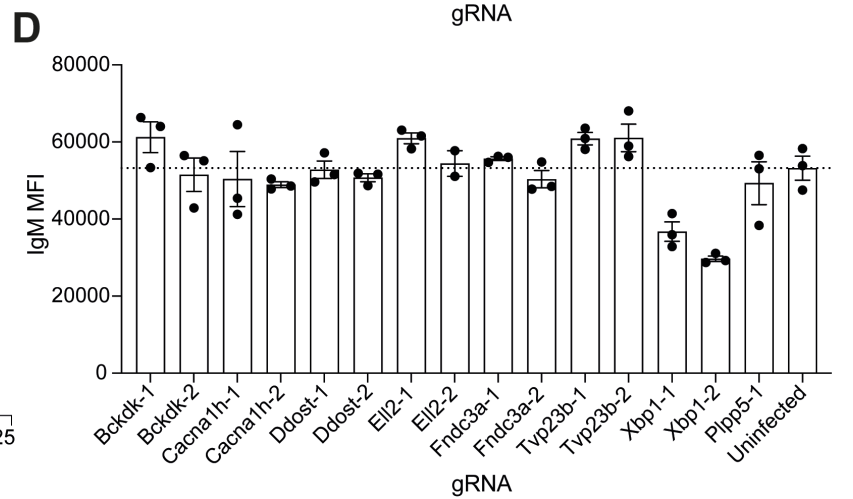
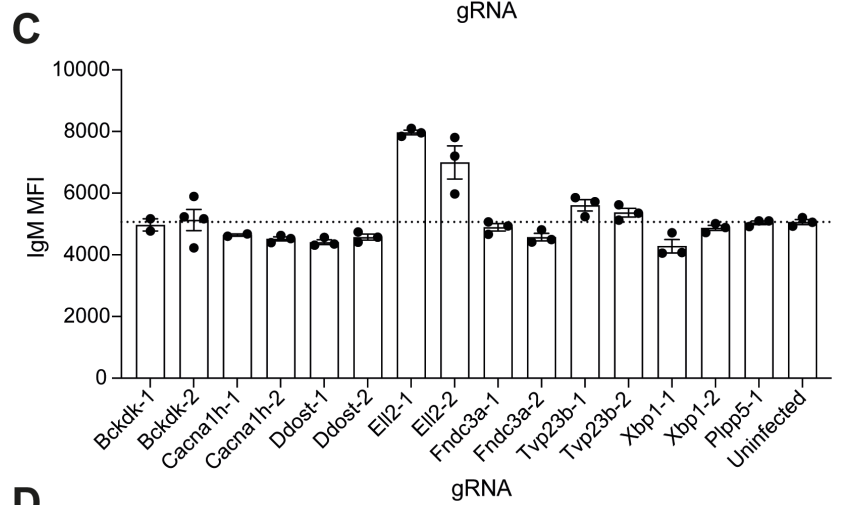
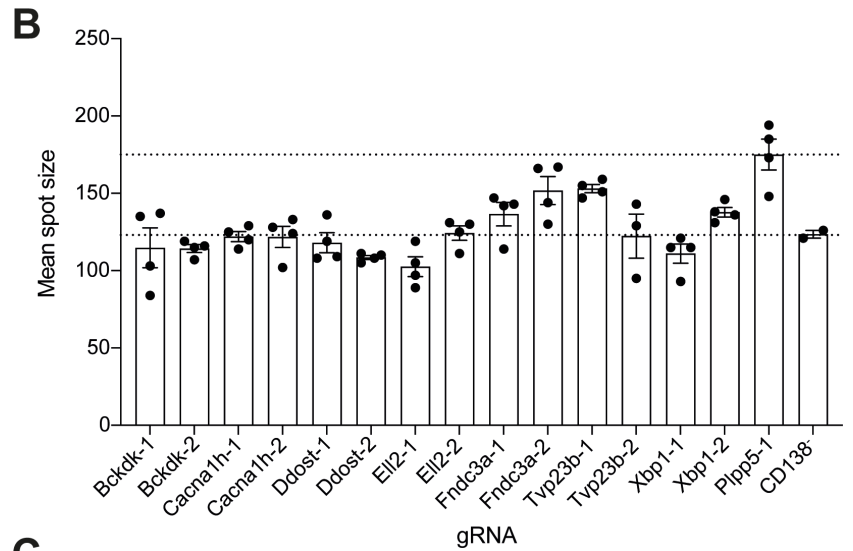
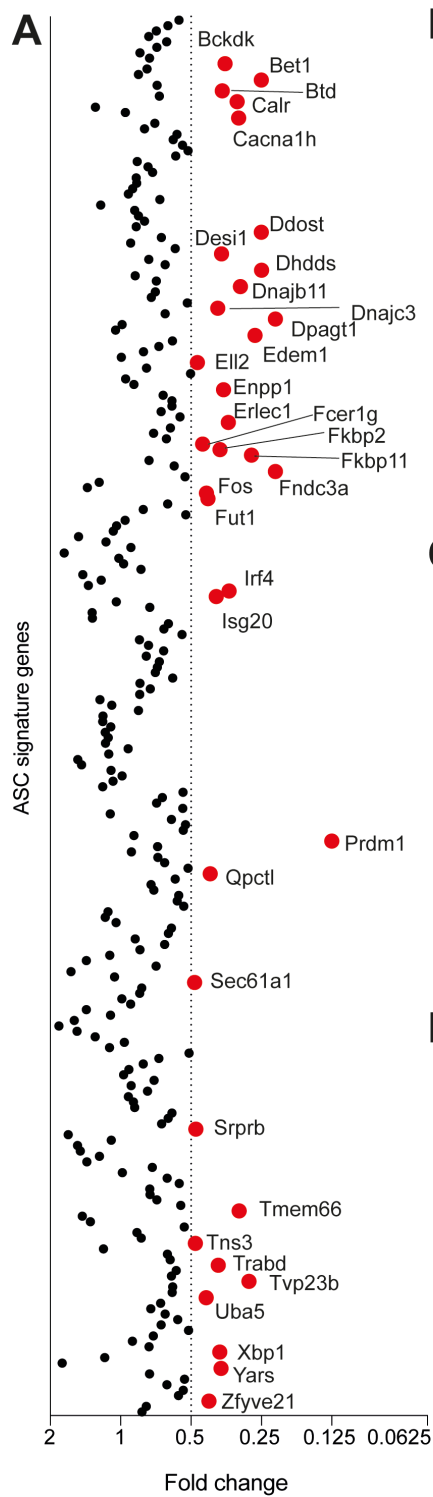


Figure 3.10 Identification of genes influencing antibody secretion

(A) Experimental workflow described in **Figure 3.1**. The concentration of IgM in the culture supernatant was measured by ELISA and normalized to the live cell number. Each data point represents the average fold change in IgM secretion per cell for both gRNAs targeting a particular gene relative to the untransduced controls on the same assay plate. Genes with a fold change of 0.5 or lower are labelled and highlighted in red. Data points represent the mean of 2 independent gRNAs from 2 replicate experiments. (B) LPS activated Cas9 B cells were transduced with the indicated gRNAs. At 2 days post-transduction, BFP⁺ cells were sorted, recultured for 24 hours and transferred to ELISpot plates. CD138⁺ activated Cas9 B cells were also added to the ELISpot plates. Each data point represents the average spot size for each well. (C-D) LPS activated Cas9 B cells were transduced with the indicated gRNAs and cultured for a further 3 days in LPS. Mean fluorescence intensity (MFI) of (C) IgM on the plasma membrane, or (D) total IgM (detected by sequential extracellular and intracellular labelling with the same antibody) of gRNA transduced cells. Representative of 3 independent experiments.

The genes whose disruption specifically impaired antibody secretion can be segregated into several groups; genes known to be required for antibody secretion (*Xbp1*, *Ell2*), genes involved in protein folding and ERAD (*Calr*, *Dnajb11*, *Edem1*, *Erlec1*, *Srprb*), genes involved in post-translational modifications (*Ddost*, *Dhdds*, *Dpagt1*, *Fut1*), and other genes or genes with an unknown function (*Bckdk*, *Bet1*, *Btd*, *Cacna1h*, *Dnajc3*, *Enpp1*, *Fcer1g*, *Fkbp2*, *Fkbp11*, *Fndc3a*, *Fos*, *Isg20*, *Qpctl*, *Tmem66*, *Tns3*, *Trabd*, *Tvp23b*, *Uba5*, *Yars*, *Zfyve21*). To confirm that these genes were influencing antibody secretion, I performed ELISpot assays on selected targets. Consistent with previous experiments, naïve B cells were activated with LPS and transduced with gRNAs targeting *Bckdk*, *Cacna1h*, *Ddost*, *Ell2*, *Fndc3a*, *Tvp23b*, *Xbp1* or *Plpp5*. At 48 hours post-transduction, BFP⁺ cells were sorted, returned to culture for 24 hours to recover and then transferred to ELISpot plates. Samples were compared to untransduced CD138⁺ activated B cells, which do not secrete antibody, and *Plpp5* targeted cells, which have normal antibody secretion. As expected, targeting *Ell2* or *Xbp1* caused a reduction in spot size, to a similar level as the CD138⁺ activated B cells (**Figure 3.10b**). Targeting *Bckdk*, *Cacna1h*, *Ddost*, *Fndc3a* or *Tvp23b* also reduced the average spot size to a similar extent, confirming the screen results that these genes are required for antibody secretion.

There are multiple stages in the antibody secretion process that these genes may be either directly or indirectly regulating, including transcriptional control of secretory immunoglobulin transcripts, protein synthesis and transport, or cellular metabolism that enables the cell to be able to constantly generate high quantities of antibody. B cells express a membrane bound form of immunoglobulin while ASCs utilise an alternate polyadenylation site, resulting in the production of a shorter secreted form (Peterson, 2011). *Ell2* is involved in the regulation of this process as it is known to promote the usage of the distal *Igh* polyadenylation site and consequentially, promote the production of transcripts encoding the secreted form of immunoglobulin (Martincic et al., 2009; Park et al., 2014). To determine whether the decrease in antibody secretion observed in this screen was due to a failure to switch from producing membrane bound to secreted forms of IgM, I examined the amount of membrane bound IgM present on the plasma membrane of cells transduced with gRNAs targeting *Bckdk*, *Cacna1h*, *Ddost*, *Ell2*, *Fndc3a*,

Plpp5, *Xbp1* or *Tvp23b*. Cells transduced with gRNAs targeting *Eli2* had increased membrane bound IgM compared to uninfected controls as shown by the increase in the mean fluorescence intensity (MFI) (**Figure 3.10c**). No other gRNAs affected the levels of membrane bound IgM present on the cell surface, suggesting that, of the examined genes, only *Eli2* was influencing the switch in polyadenylation site usage.

The decrease in IgM secretion may be the result of decreased production of IgM protein, therefore, I examined the total amount of IgM being produced by the transduced cells by sequential labelling of extracellular and intracellular IgM with the same antibody. Only cells transduced with *Xbp1* gRNAs had a reduction in total IgM levels (**Figure 3.10d**). Together, these results suggest that *Bckdk*, *Cacna1h*, *Ddost*, *Fndc3a* and *Tvp23b* are all influencing antibody secretion downstream of the transcriptional switch to producing the secreted form of immunoglobulin and downstream of the cells ability to produce high quantities of IgM and are potentially involved in the intracellular trafficking of antibody or export of antibody from the cell.

3.3. Discussion

The generation, survival and function of ASCs is critical for an effective adaptive immune response and, despite considerable work within the field to define which genes are important for ASC development and function, we still lack a complete understanding of factors that are essential for these processes. Previous work has identified a core group of expressed genes that are shared between ASC subsets, known as the ASC gene signature, and interrogating this signature provides us with the opportunity to identify novel regulators of ASC biology (Shi et al., 2015). In this thesis, I have developed a CRISPR/Cas9-mediated screening system which allows for the identification of factors essential for the differentiation, survival, proliferation and secretion capacity of primary B cells in a robust manner, without the requirements for sequencing. Using this system, I have demonstrated that within the ASC gene signature, 4 genes are required for differentiation, 10 are involved in survival or proliferation and 32 genes specifically influence antibody secretion.

3.3.1. Few elements within the ASC gene signature are essential for B cell differentiation *in vitro*.

It was surprising that few genes within the ASC gene signature appear to be essential for the differentiation process to occur *in vitro*, with only five genes reducing the proportion of CD138⁺ cells, including *Sdc1*, the gene that encodes CD138 (**Figure 3.5**). Among these five genes was *Prdm1* and *Irf4*, both extensively studied drivers of ASC formation and function (De Silva et al., 2012; Shapiro-Shelef et al., 2003; Tellier et al., 2016). *Irf4* is required for both ASC generation and survival, however, while *Prdm1* is essential for differentiation, it is dispensable for ASC survival (Tellier et al., 2016). This suggests that in the screening assay that I have used, the gRNAs were able to have an effect early enough to block differentiation from occurring.

The remaining two genes, *Sec61a1* and *Hspa5*, encode proteins important in protein translocation into the ER and ensuring correct protein folding. *Sec61a1* encodes the largest subunit of the Sec61 complex, which controls the co-translational or post-translational transport of polypeptides into the ER lumen

and peptide insertion into the ER membrane (Görlich et al., 1992; Wirth et al., 2003). This complex also regulates ER homeostasis as it acts as an ion channel that controls the rate of Ca^{2+} efflux from the ER lumen (Erdmann et al., 2011; Lang et al., 2011). *Sec61a1* has been implicated in the differentiation and survival of human ASCs as a recent study identified primary antibody deficiency patients that carried heterozygous *Sec61a1* mutations (Schubert et al., 2018). This study identified two families with *Sec61a1* mutations, one with a nonsense mutation resulting in *Sec61a1* haploinsufficiency (p.E381*) and the other with a point mutation (V85D). Interestingly, neither of these mutations resulted in changes in peripheral naïve, switched memory, unswitched memory or transitional B cell populations, however, peripheral plasmablast populations were reduced. In agreement with the reduced differentiation of *Sec61a1* targeted cells that I have shown (**Figure 3.5**), human B cells carrying *Sec61a1* mutations displayed a decreased rate of differentiation *in vitro*. This study also suggested that *Sec61a1* was required for ASC survival, as introduction of *Sec61a1* mutations into human MM cell lines resulted in terminal UPR activation and cell death. This suggests that the absence of differentiation that I observed in *Sec61a1* targeted mouse B cells is likely due to increased cell death during the differentiation process as cells try to increase their levels of antibody synthesis.

ASCs are highly dependent on the UPR and ER stress response due to their high level of antibody synthesis. *Hspa5* encodes Grp78, also known as BiP, which is a major regulator of the UPR due to its function as an ER chaperone protein (Grootjans et al., 2016). Grp78 binds to unfolded or misfolded proteins in the ER lumen to facilitate correct protein folding, however, it also binds to the ER stress sensors, Ire1a, PERK and Atf6, keeping them in an inactive state. In the absence of Grp78, the ER stress sensors activate downstream processes including the UPR, and if left unrestrained will induce cell death (Karagöz et al., 2019). It is likely that this terminal UPR activation is occurring in the *Hspa5* targeted cells resulting in the decrease in differentiation, survival and proliferation that I have observed (**Figure 3.5, 3.7 & 3.8**).

I have shown here that *Irf4*, *Prdm1*, *Sec61a1* and *Hspa5* are the only elements within the ASC gene signature that are essential for differentiation in response to

LPS stimulation *in vitro*, however, whether these are the only genes required for differentiation under any condition remains unclear. Due to the defect in the differentiation of Cas9 transgenic B cells in response to CD40L/IL-4/IL-5 stimulation (**Figure 3.4**), this screen was only performed under TI conditions, and it is possible that additional genes within the ASC gene signature may be essential for differentiation to TD stimuli. Additionally, it remains to be investigated whether the genes that did not appear to be essential for differentiation in this assay are also dispensable for differentiation *in vivo*.

3.3.2. Novel regulators of B cell proliferation and survival

This work has also identified a number of genes whose disruption altered the total live cell number by influencing survival and/or proliferation of activated B cells. While most of these genes have not been specifically linked to survival and proliferation of B cells, all genes, except *Cdv3*, have been previously been implicated in at least one of these processes in other cell types. *Rpl10*, *Rpl15*, *Rpl23a* and *Rps6* all encode ribosomal proteins and efficient protein translation is essential for cell division and survival, therefore, it is unsurprising that targeting these genes would have a dramatic effect on the live cell number. *Irf4* has previously been linked to cell division in activated B cells as it directly induces the expression of genes involved in the proliferation, including *Myc* (Shaffer et al., 2008). As discussed previously, *Hspa5/Grp78* is a major regulator of ER homeostasis and reduction in Grp78 levels can lead to the activation of the terminal UPR program and ultimately result in cell death (Karagöz et al., 2019). *Selk* and *Vcp* both encode components of the ERAD pathway, which is responsible for detecting misfolded proteins and targeting them for proteasomal degradation (Du et al., 2010; Hänzelmann et al., 2019; Lee et al., 2015; Shchedrina et al., 2011). Targeting components of this pathway increases the accumulation of misfolded proteins in the ER, which would lead to the sequestration of Grp78 and the downstream activation of the terminal UPR program. What is curious about these results, however, is that there are additional genes within the ASC gene signature (*Derl1*, *Derl3*, *Edem3*, *Herpud1*, *Hsp90b1*, *Os9* and *Sel1l*) that encode components of the ERAD pathway which did not have a clear effect on the total live cell number. *Sumo2* encodes a small ubiquitin-like

protein that can be added to other proteins to influence their stability, activity and cellular localization. This form of post-translational regulation is involved in a range of cellular processes including control of cell cycle progression (Eifler and Vertegaal, 2015). Previous work has shown that *Sumo2* deficient mouse embryonic fibroblasts have decreased cell cycling and an increase in cell death compared to WT cells (Wang et al., 2014). In agreement with these previous observations, I have shown that *Sumo2* deficient B cells also have a proliferative defect (**Figure 3.8b and 3.9**). Additionally, the anti-apoptotic protein Mcl1, is important for the survival of activated B cells and ASCs, and SUMOylation of Mcl1 significantly is reported to significantly increase its half-life, however, the specific contribution of Sumo2 is unclear (Li et al., 2020; Peperzak et al., 2013; Vikstrom et al., 2010). Therefore, the targeting of *Sumo2* is likely to also be affecting B cell survival in this system, potentially through influencing the stability of Mcl1.

The remaining gene that influenced cell number, *Cdv3*, has no previous association with proliferation or survival, or indeed any cellular process. A previous study has shown that *Cdv3* is phosphorylated by c-Abl tyrosine kinase in pro-B cell lines (Tsuchiya et al., 2003). Similarly to *c-Abl*, *Cdv3* is expressed throughout B cell development, with lowest expression in mature B cells (Brightbill and Schlissel, 2009; Li et al., 2017; Tsuchiya et al., 2003). A role for c-Abl in the regulation of *Cdv3* fits with the proliferative defect that I have observed, as c-Abl has been implicated in the regulation of B cell division. *c-Abl* deficient B cells have reduced proliferation following activation by BCR cross-linking, however, their response to LPS is largely normal (Hardin et al., 1996; Zipfel et al., 2000). I have shown here that targeting *Cdv3* impairs proliferation following LPS and CD40L stimulation (**Figure 3.8b and 3.9**). It is possible that *Cdv3* might be regulated by multiple factors downstream of mitogen receptor signalling, however, future work will be needed to place *Cdv3* into receptor signalling and cell division pathways.

B cell division and differentiation are linked processes, as the probability of a B cell undergoing differentiation increases with each division (Hasbold et al., 2004; Hodgkin et al., 1996). However, with the exception of *Irf4* and *Hspa5*, genes that altered cell division did not appear to influence differentiation. I propose the

following potential explanation for these results. While I have shown that the hits from the screen had clear effects on B cell proliferation, there is likely also a large cell death component as well, as, with the exception of *Cdv3*, all genes have previously been linked to cell survival. Not all mutations introduced by the gRNAs will be loss-of-function mutations, therefore, it is possible that the majority of the targeted cells died or had impaired proliferation and the remaining BFP⁺ cells had non-deleterious mutations and were able to divide and differentiate as normal. These non-deleterious mutations in combination with the low cell numbers for these genes may have reduced the accuracy of the differentiation readout.

3.3.3. Identification of genes that are essential for antibody secretion

This screen identified 35 genes within the ASC gene signature that are essential for antibody secretion, some of which are already known to be important for this process. Targeting *Prdm1*, *Irf4* and *Sec61a1* resulted in decreased antibody secretion as these cells were unable to undergo differentiation and therefore had not undergone the morphological and transcriptional changes required for high rates of secretory immunoglobulin production. *Xbp1* is a well-studied regulator of the UPR and is essential for plasma cells to be able to produce and secrete high quantities of antibody (Taubenheim et al., 2012; Todd et al., 2009). *Ell2* regulates the switch in usage of polyadenylation sites within the *Igh* locus to promote the production of secretory immunoglobulin transcripts (Martincic et al., 2009; Park et al., 2014).

Other genes have previously been implicated in protein processing within the ER. *Srprb* encodes a component of the signal recognition complex, which controls the co-translational targeting of polypeptides to the ER (Miller et al., 1995). *Calr* and *Dnajb11* are involved in maintaining ER homeostasis through their roles as chaperones to promote correct protein folding (Kozlov and Gehring, 2020; Shen and Hendershot, 2005). *Edem1* and *Erlec1* are components of the ERAD pathway (Cruciat et al., 2006; Oda et al., 2003). *Ddost*, *Dhdds*, *Dpagt1* and *Fut1* are all involved in post-translational modification, with *Ddost*, *Dhdds* and *Dpagt1* being required for the synthesis and attachment of *N*-linked glycosylations and *Fut1* being a factor regulating protein fucosylation (Eckert et al., 1998; Endo et al., 2003; Lin et al., 2001; Roboti and High, 2012). Correct protein glycosylation

is essential for facilitating correct protein folding, preventing protein degradation by the ERAD pathway, trafficking from the ER to the golgi, movement through the golgi and transport to the plasma membrane (Varki, 2017). All of these processes are required for antibody secretion, therefore, targeting genes regulating the addition of glycans is likely affecting at least one of these processes.

There are additional screen hits that are involved in protein secretion but are not within the ER protein processing pathway. *Bet1*, for example, is involved in trafficking of vesicles from the ER to the golgi apparatus (Hay et al., 1996; Zhang et al., 1997). But it is the remaining screen hits that do not have a known link to secretion that present the most intriguing candidates for further investigation. I selected several of these genes for more detailed analysis into which stage of the antibody secretion pathway they are regulating. Of the genes I selected for additional investigation, none influenced the transcriptional switch towards producing secretory immunoglobulin transcripts or impacted the cells ability to produce large quantities of immunoglobulin protein (**Figure 3.10c,d**). Exactly why these cells are able to produce high quantities of antibody but are unable to secrete it remains unclear.

Many of the genes identified in this screen as being essential for antibody secretion encode components of the ER protein processing pathway, with at least one hit being implicated in almost every stage of this pathway (targeting to the ER, polypeptide import, folding, *N*-linked glycosylation, recognition of misfolded proteins, and targeting of misfolded proteins for degradation) (**Figure 3.11**). It is interesting to note, however, that there are additional genes within the ASC gene signature that are implicated in this pathway that did not have a measurable effect on antibody secretion in this assay. There are several reasons as to why these additional genes did not drop out as influencing antibody production. As with any genetic screen, one of the limitations of this screen is that there is no guarantee that every gRNA will be efficient, so potentially, some of these additional genes may be essential for antibody secretion but the gRNAs targeting them are ineffective. Alternatively, these genes may be influencing antibody secretion, however, the effect that they are having is too subtle to be identified by the assay that I have used. If targeting these genes is only minimally affecting the quantity

or rate of antibody secretion, then they would not reach the cut-off of a 50 % reduction that I have used to call genes of interest. It is also possible that there is redundancy in this pathway, and that only targeting one of these genes at a time will not have any effect on the secretion process.

Another explanation as to why only a subset of genes within this pathway were identified as being essential for antibody secretion is that there may be a specific antibody secretion pathway. One potential way to investigate this possibility would be to look at the effects that all of these genes implicated in ER protein processing have on the secretion of other proteins, such as the cytokines and chemokines that ASCs are known to secrete. If the same genes are identified as influencing both antibody secretion and cytokine secretion, then it is possible that these genes are the essential elements of a general secretion process. Alternatively, if a different set of genes are identified in each screen then these screen hits then these genes may highlight a specific process for antibody secretion. Regardless, these genes may represent weak links in the antibody secretion process that could potentially be targeted therapeutically for pathologies where it would be desirable to modulate antibody secretion.

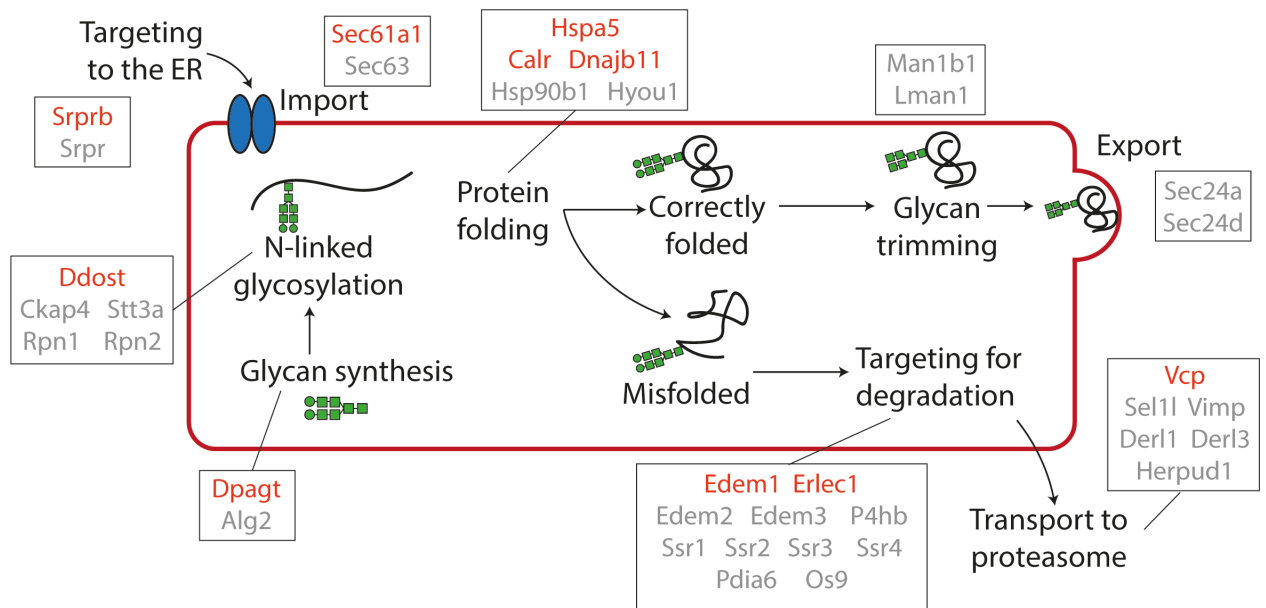


Figure 3.11 Secretion screen hits map to multiple stages of the ER protein processing pathway

Overview of the ER protein processing pathway with genes within the ASC gene signature labelled. Red indicates hits from **Figure 3.10**. Grey indicates genes within the ASC gene signature that did not reach the genes of interest cut-off. Gene function obtained from KEGG pathway: mmu04141.

3.3.4. Future directions

The majority of genes within the ASC genes signature did not appear to influence any of the parameters that I measured, however, this doesn't mean that there are not involved in these processes. In addition to the potential issues of low gRNA efficacy and gene redundancy, the assays that I have used may not be appropriate to observe the function of certain genes. These additional genes may be involved in processes that I have not investigated here, including TD differentiation responses and post-translational antibody modifications that influence antibody effector function. Furthermore, while an *in vitro* system

provides a highly controlled method to identify genes important for differentiation, it doesn't fully recapitulate the differentiation process that occurs *in vivo* (Shi et al., 2015). It would be interesting to determine whether additional genes within the ASC gene signature are dispensable for *in vitro* differentiation but required for the process *in vivo*. Additionally, it would be interesting to determine the *in vivo* effect of targeting genes that are essential for antibody secretion. It is possible that in these cells that are still able to produce high quantities of antibody yet are unable to export them from the cell, this accumulation of protein could upset cellular homeostasis and lead to cell death. This effect was not clearly observed in the *in vitro* assays that I have performed here, where differentiated cells only have a lifespan of a few days, but it is possible that the extended lifespan of these cells *in vivo* would lead to a more prolonged protein accumulation that would influence cell survival. Future work should examine this possibility, as if inhibiting antibody secretion does impact ASC survival, then these genes that I have identified as being essential for the antibody secretion process may represent novel ways to specifically target pathogenic ASC populations. Finally, an *in vitro* approach to investigating the biology of ASCs does not allow for the examination of cellular processes such as homing to the plasma cell niche or long-term plasma cell survival, therefore, future *in vivo* work is required to understand which genes within the ASC gene signature are essential for these functions.

Chapter 4. Identification of novel negative regulators of antibody secreting cell differentiation

4.1. Introduction

The differentiation of B cells into antibody secreting cells (ASCs) is an important part of the adaptive immune response to pathogens that must be tightly regulated. While the proliferation and differentiation of B cells is important for pathogen clearance, unrestrained proliferation and differentiation is associated with lymphoid malignancies and autoimmune responses. Premature ASC differentiation has consequences for the humoral immune response as cells differentiating before class-switch recombination or germinal centre formation can occur means that the antibody response generated is predominantly IgM and has relatively low antigen affinity. ASC differentiation is carefully controlled by a small collection of genes that positively or negatively regulate this process. B cell and ASC biology is regulated by two mutually antagonistic genetic networks, with B cell genes such as *Pax5* and *Bach2* repressing expression of ASC regulators, and plasma cell genes such as *Prdm1*/Blimp-1 repressing expression of B cell factors.

Several negative regulators of this process have already been identified. *Bach2* has a key role in preventing B cell differentiation by directly repressing the transcription of *Prdm1*/Blimp-1 (Ochiai et al., 2006). *Bach2*^{-/-} B cells will prematurely differentiate into ASCs at earlier timepoints and earlier divisions than WT B cells (Muto et al., 2010). *Pax5* is a master regulator of B cell biology as it is required for B cell development and for commitment to and maintenance of B cell identity (Mikkola et al., 2002; Nutt et al., 1999; Nutt et al., 1997; Urbánek et al., 1994). *Pax5* has dual roles in negatively regulating B cell differentiation, by activating the expression of B cell genes, including *Bach2* and genes involved in BCR signalling, and by repressing the expression of plasma cell genes (Delogu et al., 2006; Horcher et al., 2001; Nutt et al., 1998; Schebesta et al., 2007; Schebesta et al., 2002). *Bcl6* plays an important role in preventing premature ASC differentiation during the germinal centre response as it acts cooperatively with *Bach2* to prevent expression of *Prdm1* until affinity maturation has occurred (Huang et al., 2014; Ochiai et al., 2008). *Bcl6* also facilitates affinity maturation by repressing DNA damage responses as GC B cells introduce mutations into their immunoglobulin encoding genes to alter their affinity for antigen (Huang and Melnick, 2015).

Multiple members of the Ets family of transcription factors, Ets1, *Spi1*/PU.1 and SpiB, have been implicated in the negative regulation of B cell differentiation. Ets1 inhibits B cell differentiation in multiple ways as it is able to directly drive the expression of B cell genes, including *Pax5*, however, it can also post-translationally inhibit Blimp-1 activity, preventing the expression of plasma cell genes (John et al., 2014; John et al., 2008; Saelee et al., 2017). *Spib*^{-/-} or *Spi1*^{-/-} B cells both display slight increases in the rate of differentiation *in vitro* (Willis et al., 2017). This effect is magnified in *Spi1*^{-/-} *Spib*^{-/-} double deficient cells, suggesting a partial redundancy in the function of these closely related proteins (Willis et al., 2017). Similarly, while *Irf8* deficient B cells have a largely normal differentiation response, *Spi1*^{-/-} *Irf8*^{-/-} B cells have a substantial increase in the rate of differentiation (Carotta et al., 2014).

Several recent studies have also implicated several epigenetic mechanisms that influence the rate of B cell differentiation. *Dmnt3*^{-/-} B cells, which lack Dmnt3 dependent DNA methylation, have enhanced rates of differentiation (Barwick et al., 2018). Similarly, inhibition of Ezh2 deposition of H3K27me3 histone modifications in activated B cells accelerates the rate of differentiation (Scharer et al., 2018). Despite these advances in our understanding of how the differentiation process is controlled, it is highly likely that there are additional factors regulating the balance between differentiation or maintaining B cell identity.

Performing a genome-wide screen to systematically investigate the contribution of genes to B cell differentiation will allow the discovery of novel negative regulators of this process. The CRISPR/Cas9 system is a powerful tool for whole genome studies as it allows for high-efficiency, multiplexed gene editing. Here, I designed and performed a genome wide CRISPR/Cas9 mediated screen in primary mouse B cells for the identification of novel regulators of the B cell differentiation process. I have also used an alternative screening approach to investigate this process by designing and performing a targeted arrayed CRISPR/Cas9 screen, focusing on genes that are downregulated as naïve follicular B cells differentiate into ASCs. Through these screening techniques I have identified several genes that have not previously been implicated in ASC

differentiation. These genes present potential new avenues to therapeutically enhance the humoral immune response following vaccination.

4.2. Results

4.2.1. Design and optimization of a genome wide CRISPR/Cas9 screen to identify novel negative regulators of B cell differentiation

To identify genes that are negative regulators of the B cell differentiation process, I designed a CRISPR-Cas9 screen using primary mouse B cells. As negative regulators of the ASC differentiation process are unlikely to be found within the ASC gene signature that was used in the previous chapter, a targeted screen on the ASC gene signature would not be a suitable method to examine this process. Genome-wide knockout CRISPR-Cas9 screens are a powerful tool for the unbiased and systematic interrogation of gene function, therefore, I sought to employ this technology to investigate the differentiation process.

To demonstrate that an *in vitro* system was going to be suitable for detecting genes that repress differentiation, that is, genes that will result in an increase in the rate of differentiation when targeted, I tested this system using gRNAs directed against *Bach2*. *Bach2* is a key negative regulator of B cell differentiation, and *Bach2*^{-/-} B cells display enhanced differentiation both *in vitro* and *in vivo* (Muto et al., 2010). Using the transfection and transduction methods that are optimized for 96-well assays (**Figure 3.2**), I introduced *Bach2* gRNAs into unstimulated Cas9 B cells and cultured them with LPS and IL-4. At 4 days post-transduction, *Bach2* gRNA transduced cells displayed an approximately 4-fold increase in the rate of differentiation, as measured by the proportion of CD138⁺ cells, compared to the untransduced controls (**Figure 4.1**). These data suggests that this system will be capable of detecting genes that are negative regulators of B cell differentiation.

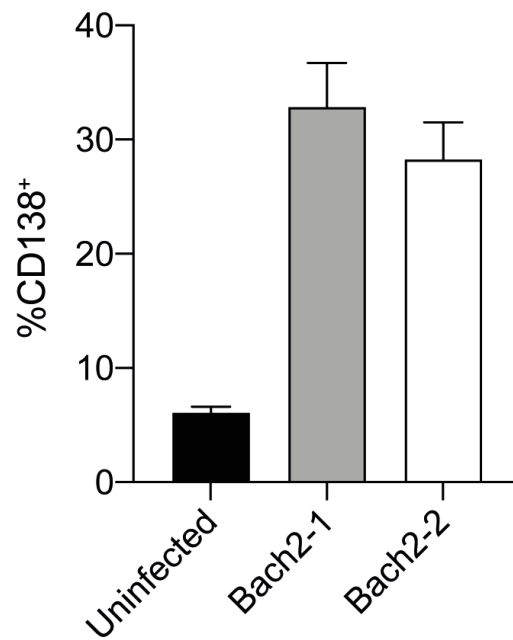


Figure 4.1 Targeting *Bach2* increases the rate of B cell differentiation *in vitro*. Naïve splenic B cells were isolated from Cas9 transgenic mice, transduced with gRNAs targeting *Bach2* and activated with LPS/IL-4. At 4 days post-transduction the proportion of CD138⁺ cells was determined by flow cytometry. Error bars represent the mean $\pm$ standard error. Representative of 2 independent experiments.

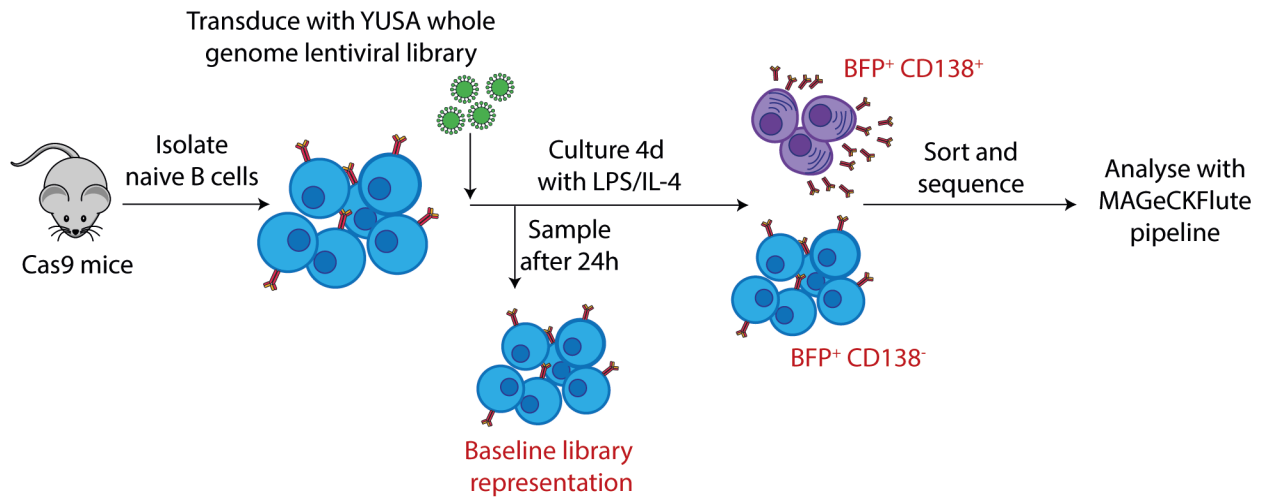


Figure 4.2 Overview of experimental workflow for genome wide CRISPR/Cas9 screen.

Naïve splenic B cells were isolated from Cas9 transgenic mice, transduced with a whole genome gRNA lentiviral library and stimulated with LPS/IL-4. All transductions were performed in triplicate wells. At 24 hours post-transduction, cells were isolated from each well (Baseline library representation) and the remaining cells were returned to culture. At 4 days post-transduction, transduced cells were sorted by flow cytometry into BFP⁺CD138⁺ and BFP⁺CD138⁻. Genomic DNA was isolated from all three populations and sequenced. Analysis of sequencing data was performed using the MAGeCK-Flute pipeline.

Next, I designed an *in vitro* screening workflow to identify negative regulators of ASC differentiation (**Figure 4.2**). Naïve splenic B cells were isolated from Cas9 transgenic mice and transduced with the Yusa mouse genome-wide CRISPR gRNA library (Koike-Yusa et al., 2014). This library contains a total of 87,897 gRNAs directed against 19,150 protein-coding genes and the lentiviral gRNA expression vector contains BFP to allow for the detection of transduced cells by flow cytometry. In contrast to the arrayed screen that I performed in **Chapter 3** where the desired transduction rate was as high as possible, whole genome screens require a lower transduction rate to ensure that only one gRNA is integrated per cell. To reduce the transduction rate, transductions were performed on unstimulated B cells (**Figure 4.3a**). After transduction, B cells were culture in LPS/IL-4 for 4 days as these stimulation conditions should induce a relatively low rate of ASC differentiation and increases above this rate should be clearly observable. These transductions were performed in triplicate with approximately 3.5 million transduced cells per replicate, meaning that each replicate has a 40-fold library coverage. At 24 hours post-transduction, approximately 1 million transduced cells were taken from each replicate to represent the baseline library representation. The 24-hour timepoint was chosen as it should allow enough time for gRNA integration into the genome but be early enough to be before gRNAs targeting essential genes are lost from the population. At 4 days post-transduction, BFP⁺CD138⁺ and BFP⁺CD138⁻ cells were sorted from each replicate and genomic DNA was isolated. For analysis of gRNAs present within each population, DNA from each replicate was PCR amplified, sequenced and reads were mapped to the gRNA library sequences. Analysis of sequencing data was performed using MAGeCK-Flute (Wang et al., 2019). In brief, this pipeline performs quality control (QC) analysis, identifies positively and negatively enriched gRNAs and genes using a maximum likelihood estimation and performs downstream functional analysis of enriched genes using gene ontology (GO) and KEGG databases. Using this method, it should be possible to identify negative regulators of differentiation and determine whether there are specific gene pathways that are involved in this process

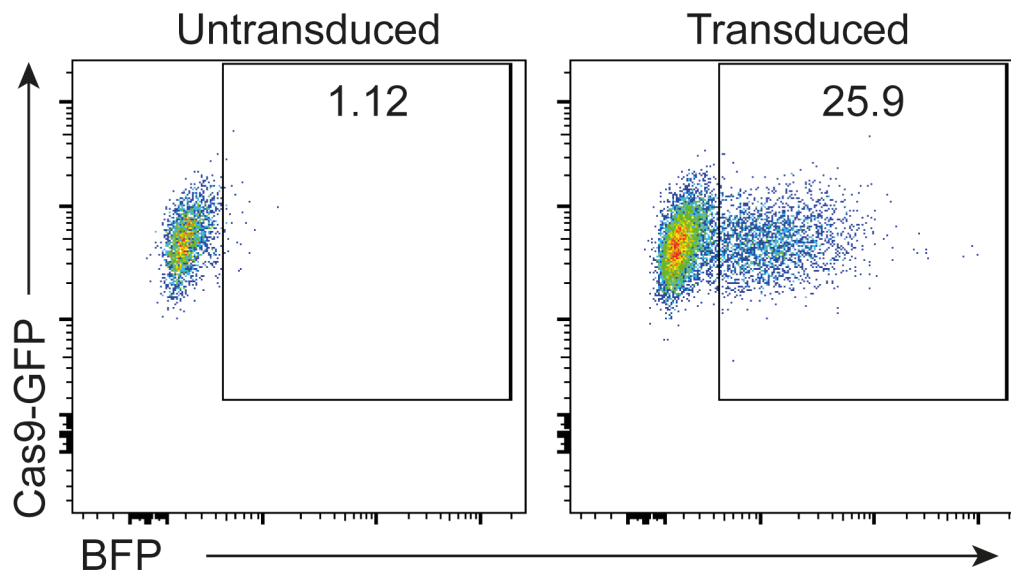


Figure 4.3 Transduction of unstimulated B cells results in relatively low transduction rates.

Naïve Cas9 B cells were transduced with a gRNA targeting Plpp5. At 48 hours post-transduction the proportion of transduced cells (BFP^+) was determined by flow cytometry. Number indicates the proportion of BFP^+ cells. Representative of 2 independent experiments.

4.2.2. Analysis of whole genome screen results using MAGeCK-Flute

The analysis of this sequencing data was performed entirely using the MAGeCK-Flute pipeline (Li et al., 2014; Wang et al., 2019). Reads were first aligned to sequences from the Yusa gRNA library and MAGeCK-VISPR performed QC analysis to determine the evenness of the library representation and sequencing quality (Li et al., 2015). All samples passed the QC checks which ensured that samples have a similar G/C content, high percentage of mapped reads, a low number of gRNAs that have zero mapped reads, and that reads from the baseline

library were evenly distributed across the gRNA library. This confirmed that there were no errors in the preparation of the lentiviral or DNA libraries and no sample contamination during library synthesis. Read counts for each gRNA were normalized to the total gRNA count for each replicate sample.

MAGeCK-MLE next calculates a beta score for each targeted gene, which is a measure of the degree of selection that occurs as a result of gene perturbation. The mean read count and variance for each gRNA is generated for each population (baseline library representation, CD138⁺ and CD138⁻). The mean read count of each of the sample groups is then compared to baseline library representation and gRNAs are ranked based on the degree of selection. From this gRNA ranking, a beta score is calculated for each gene. Genes with multiple gRNAs at the top of the ranked list have the highest beta score, representing an enrichment in that population compared to the baseline. Similarly, genes with multiple gRNAs at the bottom of the rank will have the lowest beta scores, which indicates that they are underrepresented in that population. Genes with most gRNAs in the middle of the rank will have a beta score close to zero, indicating that they have no effect in this screen. FluteMLE was then used to perform QC based on the beta scores for both the CD138⁺ and CD138⁻ population. Both conditions had a similar distribution of beta scores, indicating that it was appropriate to compare these populations in the downstream analysis (**Figure 4.4**).

Having completed the QC for the CD138⁺ and CD138⁻ populations, a differential beta score was then calculated for each gene by subtracting the beta score in CD138⁻ from the beta score in CD138⁺. Genes with a differential beta score of greater than 1 were classified as enriched in the CD138⁺ population and genes with a differential beta score of less than -1 were classified as enriched in the CD138⁻ population. *Xlr*, *Krtap4-7*, *Id2*, *Cox17*, *Rplp1*, *Med8*, *Rpl36*, *Rdh14*, *Olf1084* and *Olf1415* were the top 10 most enriched genes in the CD138⁺ population (**Figure 4.5a**). None of these genes have previously been associated with B cell or plasma cell biology. *Rpl36* and *Rplp1* encode ribosomal proteins, which would be predicted to be essential for cell survival, not negative regulators

of differentiation. Furthermore, *Olf1084* and *Olf1415* encode olfactory receptors and *Krtap4-7* encodes a keratin associated protein, and neither of these gene groups are expressed in immune cells. Curiously, known negative regulators of B cell differentiation, such as *Bach2* and *Ets1*, were not among these most enriched genes. The genes that were enriched in the CD138⁻ population were similarly unusual (**Figure 4.5b**). I predicted that genes that are essential for the differentiation process, such as *Prdm1* and *Irf4*, would be among the top enriched genes, however, the top 10 enriched genes did not contain any known positive regulators of differentiation.

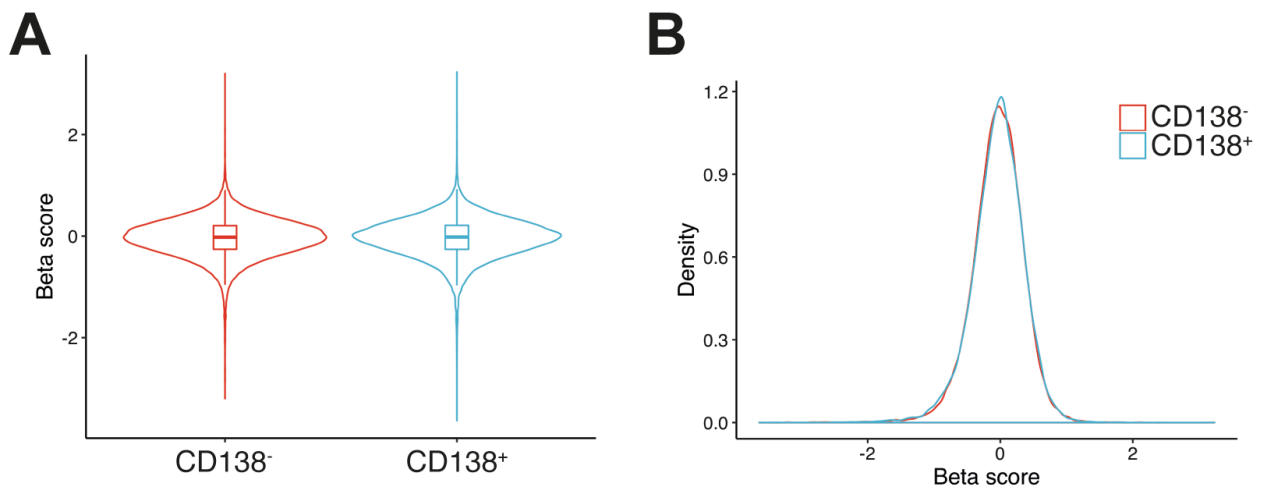


Figure 4.4 Quality control assessment of CRISPR/Cas9 screen data by FluteMLE

Experimental workflow described in **Figure 4.2**. Beta scores are calculated by the change in the representation of gRNAs targeting a gene in the sample population compared to the baseline library representation. Analysis of gRNA beta scores within the BFP⁺CD138⁺ (CD138⁺) and BFP⁺CD138⁻ (CD138⁻) populations was performed by FluteMLE. Distributions of beta scores from the CD138⁺ and CD138⁻ populations displayed as (A) violin plots and (B) histograms.

To gain a clearer understanding of the effects that targeting these genes was having in both the CD138⁺ and CD138⁻ populations, I plotted the beta score for each population against each other (**Figure 4.5c**). Beta scores for genes that are positive regulators of differentiation would be expected to fall below -1 on the CD138⁺ beta score axis, and negative regulators would be expected to fall above 1. However, known positive or negative regulators of differentiation were not found among the top differential genes.

It was possible that the known regulators of differentiation were enriched in the appropriate populations, but their effects were less pronounced than the genes identified as the top hits. To investigate this possibility, I labelled known positive and negative regulators of differentiation and examined their beta scores in each population (**Figure 4.6**). I also labelled genes that I had previously shown have a strong effect on the live cell number in this system, either through affecting proliferation or survival (**Figure 3.8**), and these genes would be expected to have beta scores of less than -1 in both populations. This revealed that *Prdm1* did fall into the expected range (CD138⁺ beta score less than -1), indicating that guides targeting it were largely absent from the differentiated population. *Rpl23a* also fell into the expected range (beta score less than -1 in both populations) as it strongly affected cell number and therefore should be lost from both populations. Concerningly, many of these genes, *Sec61a1*, *Vcp*, *Selenok/Selk*, *Eed*, *Sus12*, *Ezh2*, *Sumo2*, *Sdc1*, *Irf4* and *Hspa5*, had beta scores close to zero in both populations, meaning that they did not affect either differentiation or cell number. As these positive controls were not identified as hits, and the genes that were identified as hits did not make biological sense, I concluded that the data from this whole genome screening approach did not yield a meaningful assessment of the negative regulators of ASC differentiation.

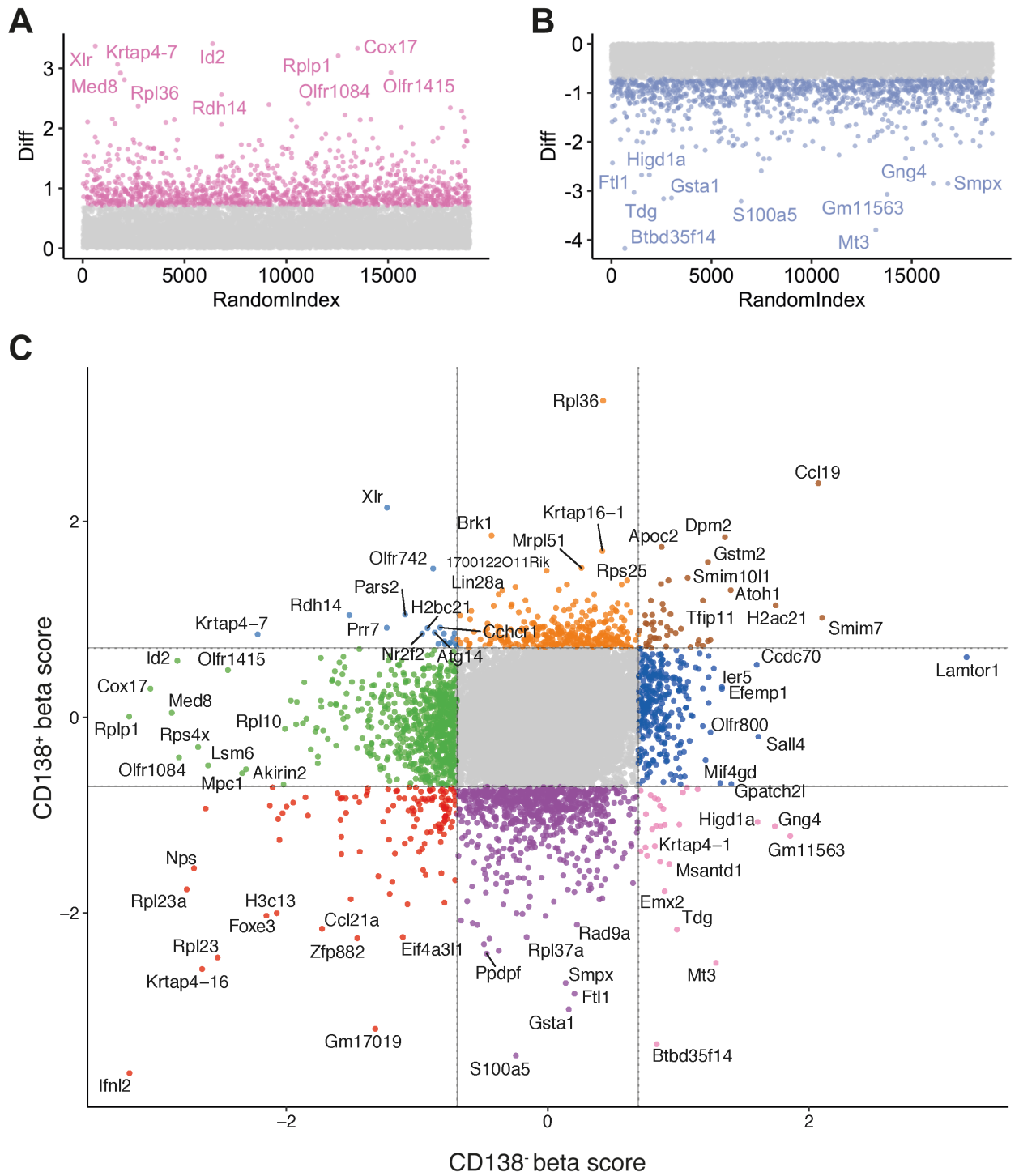


Figure 4.5 Identification of differentially enriched genes by MAGeCK-Flute.

*Experimental workflow described in **Figure 4.2** and beta scores calculations described in **Figure 4.4**. (A-B) Differential beta scores (Diff) were calculated by subtracting the beta score for each gene in CD138⁻ from the beta score in CD138⁺. (A) Genes with a differential beta score >0 are assigned a random index number to randomly distribute them across the x-axis. Genes enriched in the CD138⁺ population (differential beta score ≥ 1) are shown in pink with the 10 genes with the highest scores labelled. (B) Genes with a differential beta score < 0 are assigned a random index number to randomly distribute them across the x-axis. Genes enriched in the CD138⁻ population (differential beta score ≤ -1) are shown in blue with the 10 genes with the lowest scores labelled. (C) This plot shows the beta score for each gene in the CD138⁺ and CD138⁻ population. Horizontal and vertical lines indicate the mean $\pm$ one standard deviation of CD138⁺ and CD138⁻ beta scores respectively. Genes are coloured based on their selection in each population. Positive in both (brown), positive in CD138⁻ only (dark blue), positive in CD138⁻ and negative in CD138⁺ (pink), negative in CD138⁺ only (purple), negative in both (red), negative in CD138⁻ only (green), positive in CD138⁺ and negative in CD138⁻ (light blue) and positive in CD138⁺ only (orange). The top 10 genes within each group are labelled with the gene name.*

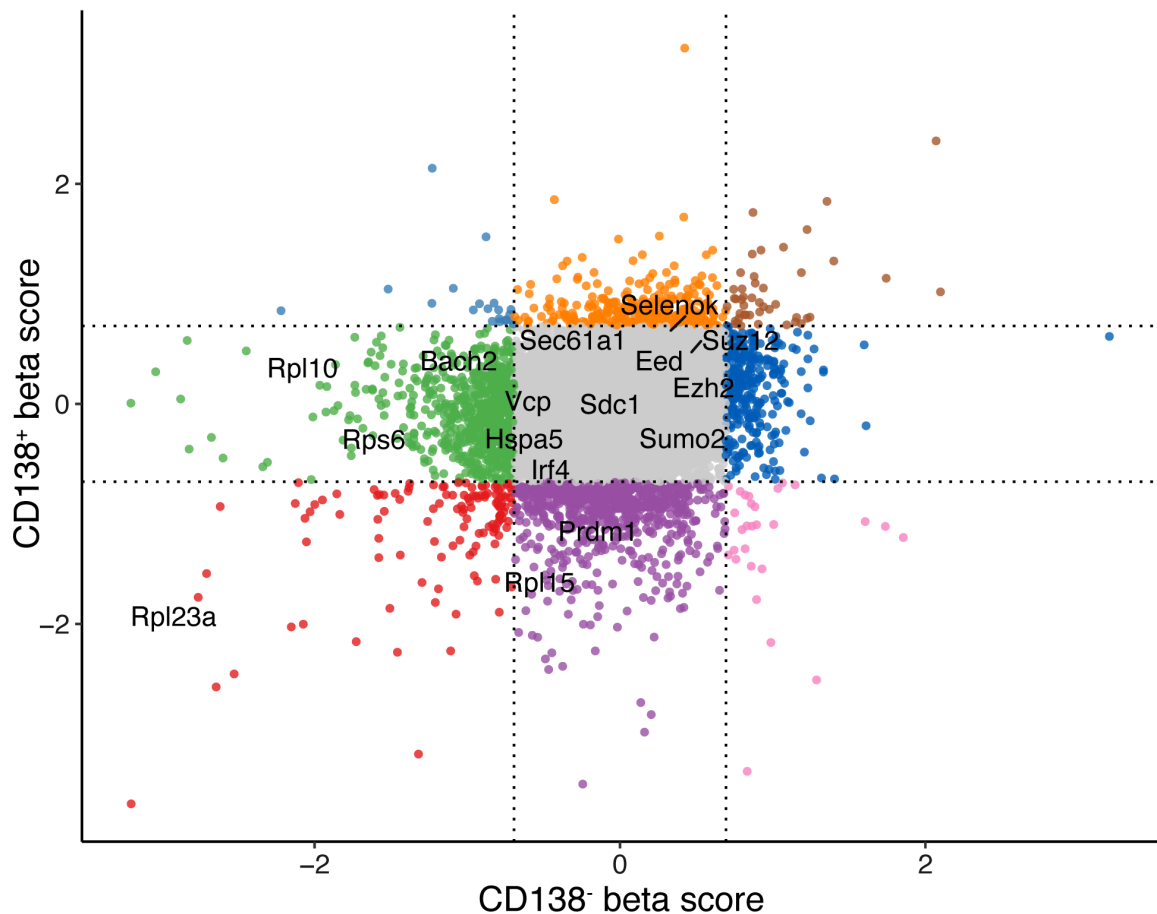


Figure 4.6. Changes in enrichment of genes of interest

*This plot is the same as **Figure 4.5c**, however, the top genes in each group are not labelled. Labels indicate genes of interest with known roles in B cell differentiation, proliferation or survival.*

Figure 4.7 Expression of arrayed screen gene set in B cell and ASC populations
Expression profile of 155 genes that are downregulated in ASC populations compared to FoB samples ($FDR \leq 0.05$, fold-change ≥ 3 , FPKM in FoB samples ≥ 32). Heatmap is colour-coded according to Z score. Data were obtained from Shi et al. Nature Immunology (2015).

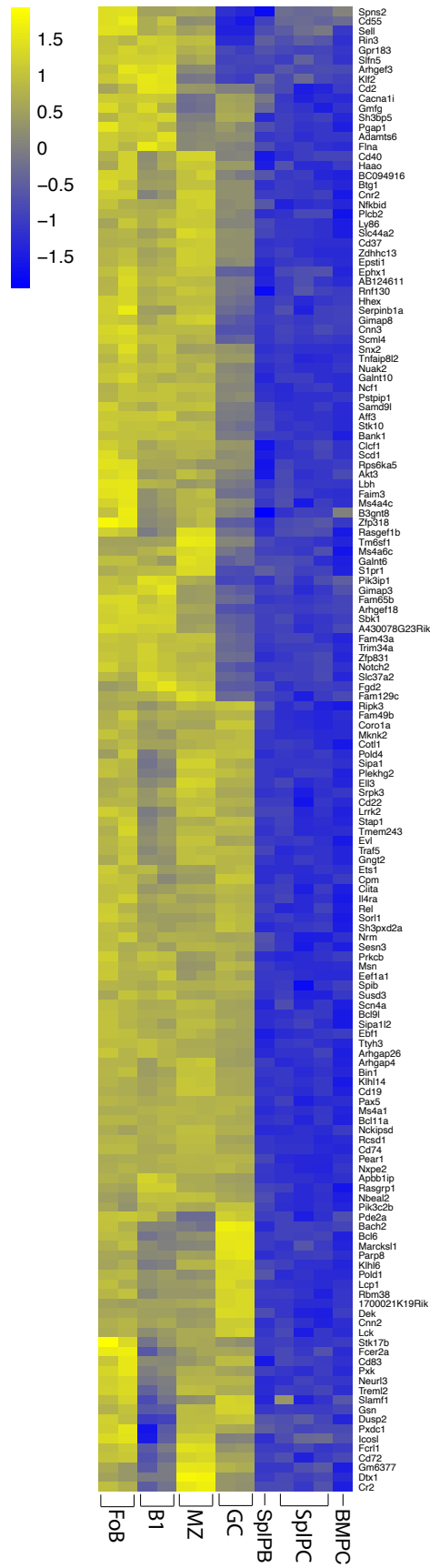


Table 4.1 Genes included in the targeted gRNA library

Gene name	MGI ID	Gene name	MGI ID
1700021K19Rik	MGI:1915160	Ciita	MGI:108445
A430078G23Rik	MGI:2442135	Clcf1	MGI:1930088
AB124611	MGI:3043001	Cnn2	MGI:105093
Adamts6	MGI:1347348	Cnn3	MGI:1919244
Aff3	MGI:106927	Cnr2	MGI:104650
Akt3	MGI:1345147	Coro1a	MGI:1345961
Apbb1ip	MGI:1861354	Cotl1	MGI:1919292
Arhgap26	MGI:1918552	Cpm	MGI:1917824
Arhgap4	MGI:2159577	Cr2	MGI:88489
Arhgef18	MGI:2142567	Dek	MGI:1926209
Arhgef3	MGI:1918954	Dtx1	MGI:1352744
B3gnt8	MGI:2385269	Dusp2	MGI:101911
Bach2	MGI:894679	Ebf1	MGI:95275
Bank1	MGI:2442120	Eef1a1	MGI:1096881
BC094916	MGI:3584522	Ell3	MGI:2673679
Bcl11a	MGI:106190	Ephx1	MGI:95405
Bcl6	MGI:107187	Epsti1	MGI:1915168
Bcl9l	MGI:1933114	Ets1	MGI:95455
Bin1	MGI:108092	Evl	MGI:1194884
Btg1	MGI:88215	Faim3	MGI:1916419
Cacna1i	MGI:2178051	Fam129c	MGI:3686743
Cd19	MGI:88319	Fam43a	MGI:2676309
Cd2	MGI:88320	Fam49b	MGI:1923520
Cd22	MGI:88322	Fam65b	MGI:2444879
Cd37	MGI:88330	Fcer2a	MGI:95497
Cd40	MGI:88336	Fcrl1	MGI:2442862
Cd55	MGI:104850	Fgd2	MGI:1347084
Cd72	MGI:88345	Flna	MGI:95556
Cd74	MGI:96534	Galnt10	MGI:1890480
Cd83	MGI:1328316	Galnt6	MGI:1891640

Gene name	MGI ID
Gimap3	MGI:1932723
Gimap8	MGI:2685303
Gm6377	MGI:3647255
Gmfg	MGI:1927135
Gngt2	MGI:893584
Gpr183	MGI:2442034
Gsn	MGI:95851
Haa0	MGI:1349444
Hhex	MGI:96086
Icosl	MGI:1354701
Il4ra	MGI:105367
Klf2	MGI:1342772
Klhl14	MGI:1921249
Klhl6	MGI:2686922
Lbh	MGI:1925139
Lck	MGI:96756
Lcp1	MGI:104808
Lrrk2	MGI:1913975
Ly86	MGI:1321404
March1	MGI:1920175
Marcksl1	MGI:97143
Mknk2	MGI:894279
Ms4a1	MGI:88321
Ms4a4c	MGI:1927656
Ms4a6c	MGI:2385644
Msn	MGI:97167
Nbeal2	MGI:2448554
Ncf1	MGI:97283
Nckipsd	MGI:1931834
Neurl3	MGI:2429944
Nfkbid	MGI:3041243

Gene name	MGI ID
Notch2	MGI:97364
Nrm	MGI:2146855
Nuak2	MGI:1921387
Nxpe2	MGI:1925502
Parp8	MGI:1098713
Pax5	MGI:97489
Pde2a	MGI:2446107
Pear1	MGI:1920432
Pgap1	MGI:2443342
Pik3c2b	MGI:2685045
Pik3ip1	MGI:1917016
Plcb2	MGI:107465
Plekhg2	MGI:2141874
Pold1	MGI:97741
Pold4	MGI:1916995
Prkcb	MGI:97596
Pstpip1	MGI:1321396
Pxdc1	MGI:1914145
Pxk	MGI:1289230
Rasgef1b	MGI:2443755
Rasgrp1	MGI:1314635
Rbm38	MGI:1889294
Rcsd1	MGI:2676394
Rel	MGI:97897
Rin3	MGI:2385708
Ripk3	MGI:2154952
Rnf130	MGI:1891717
Rps6ka5	MGI:1920336
S1pr1	MGI:1096355
Samd9l	MGI:1343184
Sbk1	MGI:2135937

Gene name	MGI ID
Scd1	MGI:98239
Scml4	MGI:2446140
Scn4a	MGI:98250
Sell	MGI:98279
Serpinb1a	MGI:1913472
Sesn3	MGI:1922997
Sh3bp5	MGI:1344391
Sh3pxd2a	MGI:1298393
Sipa1	MGI:107576
Sipa1l2	MGI:2676970
Slamf1	MGI:1351314
Slc37a2	MGI:1929693
Slc44a2	MGI:1915932
Slfn5	MGI:1329004
Snx2	MGI:1915054
Sorl1	MGI:1202296
Spib	MGI:892986
Spns2	MGI:2384936
Srpk3	MGI:1891338
Stap1	MGI:1926193
Stk10	MGI:1099439
Stk17b	MGI:2138162
Susd3	MGI:1913579
Tm6sf1	MGI:1933209
Tmem243	MGI:3606159
Tnfaip8l2	MGI:1917019
Traf5	MGI:107548
Trem12	MGI:2147038
Trim34a	MGI:2137359
Ttyh3	MGI:1925589
Zdhhc13	MGI:1919227

Gene name	MGI ID
Zfp318	MGI:1889348
Zfp831	MGI:3641861

4.2.3. Design of an arrayed targeted CRISPR/Cas9 screen to identify novel negative regulators of B cell differentiation

After determining that using a genome-wide screening CRISPR gRNA library with this method of *in vitro* differentiation of primary B cells was not a suitable approach to identify negative regulators of differentiation, I next considered using an arrayed target approach, similar to the one I had previously used (**Chapter 3**). However, negative regulators of the differentiation process are unlikely to be within the ASC gene signature that the previous screens targeted. To select the most appropriate genes to target, I reanalysed the RNA-seq data that the ASC gene signature was identified from and defined a gene list using similar criteria, however, this time focusing on the genes that were downregulated through the differentiation process (Shi et al., 2015). Genes that had a 3-fold higher expression in Follicular B (FoB) samples compared to all ASC subsets (≤ 0.05 FDR, ≥ 32 FPKM in FoB samples) were included in the targeted gene list (**Figure 4.7, Table 4.1**).

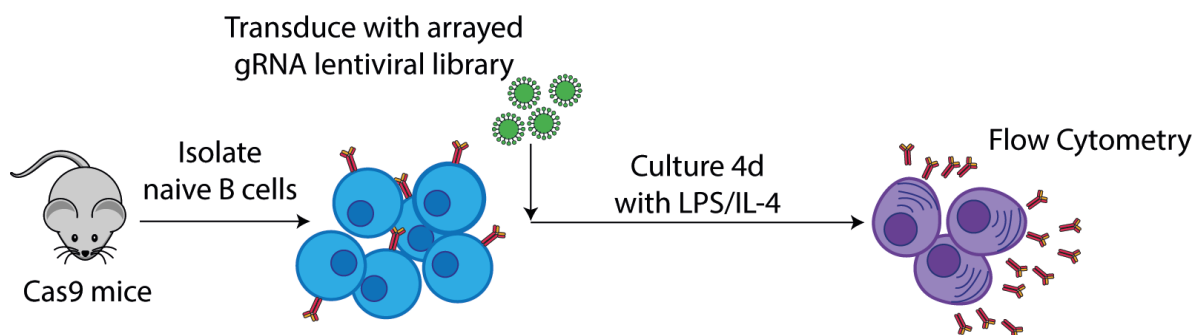


Figure 4.8 Overview of experimental workflow for targeted arrayed CRISPR/Cas9 screen

Naïve splenic B cells were isolated from Cas9 expressing transgenic mice and transduced with an arrayed lentiviral gRNA library. Following transduction, cells were cultured in LPS/IL-4 for 4 days before analysis by flow cytometry.

I next synthesized an arrayed gRNA lentiviral library using the protocol I had previously optimized (**Chapter 3**). Transfections were performed in batches of 96 gRNAs in parallel and at 48 hours post-transfection lentivirus containing supernatant was transferred to fresh 96-well plates and stored at -80 °C until use. Every library plate generated contained at least 2 empty wells to serve as negative controls. All transfection efficiencies were checked by flow cytometry and no gRNAs had a transfection efficiency of less than 80 %.

A screen for negative regulators was then performed on the selected gene set using the arrayed lentiviral gRNA library (**Figure 4.8**). Naïve splenic B cells were isolated from Cas9 transgenic mice and transduced with the arrayed lentiviral gRNA library. Following transduction, B cells were stimulated with LPS/IL-4 and cultured for 4 days before analysis. The effect of each gRNA on differentiation was determined by the proportion of BFP⁺ cells that expressed CD138. The cut-off for genes of interest was arbitrarily set as having at least a 2-fold increase in the proportion of differentiated cells, relative to the uninfected control on each assay plate (**Figure 4.9**). As was expected from previous work, and in agreement with the validation experiments, targeting *Bach2* resulted in a clear increase in the proportion of differentiated cells. The other gRNAs that resulted in a clear increase in differentiation were directed against *Ets1*, *Pax5* and *Pold1*. I also observed a more modest effect in targeting *AB124611*, *Arhgef18*, *A430078G23Rik*, *Fam43a*, *Ripk3*, *Rnf130* and *Rsp6ka5*. Interestingly, there was an additional gene, *Eef1a1*, which appeared to be a positive regulator of differentiation as targeting this gene resulted in a decrease in the proportion of CD138⁺ cells. These results show that an arrayed screening approach may be a more appropriate method to identify regulators in primary cells as it is sensitive enough to detect small changes in the measured parameters.

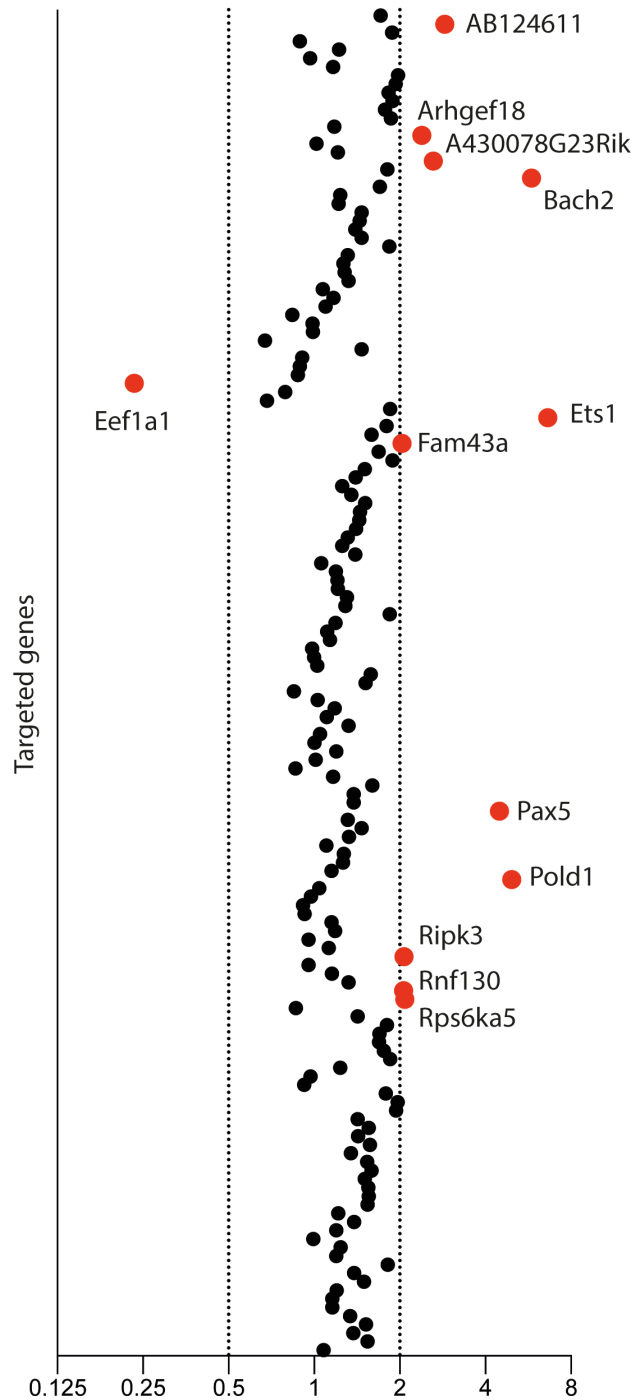


Figure 4.9 Identification of negative regulators of B cell differentiation.

Experimental workflow is described in **Figure 4.8**. Each data point represents the average fold change in the proportion of CD138⁺ cells for both gRNAs targeting a particular gene relative to the untransduced controls on the same assay plate. Genes with a fold change of < 0.5 or > 2 are labelled and highlighted in red. Data points represent the mean of 2 independent gRNAs from 2 replicate experiments.

4.3. Discussion

The differentiation of B cells into ASCs is a tightly controlled process that requires the silencing of the B cell transcriptional program and the activation of the ASC program. I have demonstrated that targeting the known negative regulator, *Bach2*, in primary mouse B cells *in vitro* results in a clear increase in the rate of differentiation (**Figure 4.1**), therefore, an *in vitro* system should be suitable to identify additional negative regulators of this process. Here I have utilised 2 different CRISPR/Cas9 screening techniques to identify novel negative regulators of the differentiation process.

4.3.1. A genome wide pooled CRISPR/Cas9 screen failed to identify novel regulators of B cell differentiation

I first attempted to uncover novel negative regulators of differentiation using an unbiased genome-wide screening approach. Unfortunately, I believe that this approach was unsuccessful. There are several components required for a successful whole genome screen: effective gRNAs with minimal off-target effects, appropriate library coverage and depth of sequencing, and strong selection pressure. In this section I will discuss each of these issues in the context of the genome-wide screen I have performed.

Performing a screen on primary cells means that a balance must be found between ensuring optimal library coverage and the practicality of culturing such large numbers of primary cells. Typical genome-wide screens require a library coverage of between 50-200-fold for optimal background noise reduction. For this whole genome screen, I selected a library coverage of 50-fold per replicate, with a total coverage of 150-fold for each sample type. Based on the QC analysis of my sequencing data and baseline library representation (**Figure 4.3**), both the library coverage and sequencing depth that I have used was appropriate, therefore, it is unlikely that poor coverage was the problem with this screen.

Off-target activity is variable depending on both the gRNA sequence and the conditions of the experiment and has the potential to result in false positive results. Off-target activity is particularly of concern for genes that only have one or two gRNAs targeting them within the gRNA library. Off-target effects may

explain why the analysis shows a clear enrichment in genes such as genes encoding ribosomal proteins like *Rpl36* and *Rplp1*, which are essential genes and should be lost from both the differentiated and undifferentiated populations, appeared among the top enriched genes in the CD138⁺ cells. The Yusa genome-wide library was designed to have minimal off-target effects, however, it was designed before the rules dictating gRNA on-target activity were fully identified, meaning that it contains a higher proportion of gRNAs that are predicted to have poor on-target efficacy than more recently designed libraries (Doench et al., 2016; Koike-Yusa et al., 2014).

Poor on-target efficacy is a key limitation of this screen and will result in false negative results, that is genes which are true regulators appear to have no effect as the gRNAs that target them are ineffective. The MAGeCK pipeline is able to identify and exclude low efficiency gRNAs, however, if most gRNAs directed against the same gene are ineffective then this will not be possible, and the analysis will show that the gene has no effect. This may explain why many of the known regulators of ASC differentiation do not appear to be hits in this analysis. From previous work investigating the genetic networks that control ASC differentiation, and from the arrayed screen that I performed (**Chapter 3**), it is known that targeting *Irf4*, *Prdm1*, *Hspa5* or *Sec61a1* should completely block differentiation, however, gRNAs targeting all of these genes were identified in the differentiated population. Most concerningly, gRNAs targeting *Sdc1*, which encodes CD138, were also detected in the CD138⁺ population. Similarly, several genes that influence cell number (*Selk*, *Sumo2*, *Hspa5*, *Irf4*, *Vcp*) (**Chapter 3**), were not lost from one or both of the populations, as would be expected (**Figure 4.6**). Furthermore, gRNAs targeting known negative regulators of ASC differentiation including *Bach2*, were not found to be enriched in the differentiated population. Together, this suggests that many of the gRNAs targeting known regulators are ineffective. This issue is unlikely to be restricted to known regulators, therefore, it is likely that gRNAs targeting novel negative regulators would be similarly inefficient and that these genes would not be able to be identified in this screen.

The final issue that potentially reduced the effectiveness of this screen is the strength of the selection pressure. Whole genome screens require a very strong selection pressure to see clear enrichment or loss of gRNAs within the sample populations. While the culture conditions that I selected induce a weak differentiation response, there is still some differentiation that occurs. It is possible that gRNAs targeting genes that are not negative regulators of differentiation are present in the differentiated population by chance. Although gRNAs targeting negative regulators should be enriched, there may be too many gRNAs included by random chance to clearly see this enrichment.

It is likely that any or all of these issues are the reason that the whole genome CRISPR screen did not yield meaningful results. The on-target efficacy can be increased and off-target effects minimalised by using a different genome-wide CRISPR gRNA library. There are gRNA libraries available that have been designed since the rules that dictate gRNA effects have been elucidated and consequentially, these libraries will have better on-target effects. If a whole genome screen is to be employed in the future to investigate this process, it would be beneficial to assess whether a different library may be more appropriate. Changing the culture condition to a system that has reduced baseline differentiation would increase the selection pressure of this assay, however, which alternate condition should be used is not clear. Culturing B cells in LPS and IL-4 already induces a relatively low rate of differentiation. CD40L and IL-4 cultures contain even fewer differentiated cells, however, Cas9 transgenic B cells display altered differentiation to CD40L culture conditions (**Figure 3.4**). Stimulation with CpG alone does not induce any B cell differentiation so theoretically this system should provide the strongest selection pressure. However, it is not clear if targeting a negative regulator would be sufficient to induce differentiation, or whether with this culture condition B cells will never be able to differentiate as expression of positive regulators will not be induced.

4.3.2. A targeted arrayed CRISPR/Cas9 screen reveals novel negative regulators of B cell differentiation

After determining that the genome-wide screening approach I attempted was unsuccessful, I hypothesised that an arrayed screen, similar to the one that I performed to identify positive regulators of differentiation (**Chapter 3**), may be a more appropriate method to use. Arrayed screening provides an extremely sensitive method of detecting changes in the rate of differentiation, and with no requirements for library coverage or sequencing depth, it overcomes many of the technical limitations that I encountered with the pooled screen. However, performing a genome-wide arrayed screen does have limitations, most notably obtaining sufficient numbers of naïve B cells and the limited throughput of the flow cytometry readout, therefore, I elected to perform a targeted screen. To select the appropriate gene set for investigation, I reanalysed the transcriptomic data that the ASC gene signature was defined from (Shi et al., 2015). Known negative regulators of B cell differentiation such as *Pax5* and *Bach2* have higher expression in B cells than in ASCs, therefore, I hypothesised that additional negative regulators would most likely be within the set of genes whose expression is decreased in ASC subsets compared to naïve follicular B cells. Based on this analysis, I selected 155 genes to interrogate with a targeted arrayed screen. The screening approach that I used was a modified version of the assay that I used to detect positive regulators of differentiation, as I lowered rates of differentiation by adding IL-4 to the cell cultures and introduced the gRNAs into unstimulated cells to allow the gRNAs to have an effect at an earlier timepoint (**Figure 4.8**). This approach identified 11 genes that increased the rate of differentiation and 1 that decreased differentiation (**Figure 4.9**). Among the genes that resulted in the largest increase in the proportion of CD138⁺ cells were the known negative regulators of B cell differentiation, *Bach2*, *Pax5* and *Ets1*.

Bach2 is a known negative regulator of B cell differentiation, as it represses the expression of *Prdm1*/Blimp-1. It has been shown previously that *Bach2*^{-/-} B cells display increased rates of differentiation, and this increase was evident in my screen results (Muto et al., 2010; Muto et al., 2004; Ochiai et al., 2006). *Ets1* has a dual role in the negative regulation of B cell differentiation as it can directly activate *Pax5* expression, and it can post-translationally inhibit Blimp-1 activity

(John et al., 2014; John et al., 2008; Saelee et al., 2017). Ectopic expression of *Ets1* reduces the rate of differentiation (John et al., 2014; John et al., 2008; Saelee et al., 2017). Conversely, *Ets1*^{-/-} B cells display enhanced differentiation *in vitro* under multiple stimulation conditions, and *Ets1*^{-/-} mice have expanded ASC populations and increased serum antibody titres (Barton et al., 1998; Bories et al., 1995; John et al., 2014; Saelee et al., 2017; Wang et al., 2005). The increased rate of differentiation observed in my screen agrees with previous reports that *Ets1*^{-/-} B cells display enhanced differentiation *in vitro*.

Pax5 is a master regulator of B cell biology as it is required for both commitment to the B lymphocyte lineage and maintaining mature B cell identity (Nutt et al., 1999; Nutt et al., 1997; Rolink et al., 1999). *Pax5* expression is maintained in activated B cells until it is downregulated in one of the earliest stages of the differentiation process (Kallies et al., 2007). It was previously thought that the downregulation of *Pax5* is essential for differentiation to occur, however, recent work has challenged this dogma, showing that mouse B cells that cannot downregulate *Pax5* display a largely normal differentiation response (Liu et al., 2020). Loss of *Pax5* expression has been shown to have different effects on naïve B cells, activated B cells and B cell lines. Deletion of *Pax5* in naïve mature B cells results in cells reverting to an earlier progenitor state and they are no longer committed to the B cell lineage (Cobaleda et al., 2007; Mikkola et al., 2002). Loss of *Pax5* expression in the chicken B cell line DT40 results in some aspects of ASC differentiation, however, the knockdown of *Pax5* expression in activated mouse B cells using RNAi does not alter the rate of differentiation (Kometani et al., 2013; Nera et al., 2006). RNAi can result in incomplete gene silencing and reduced levels of *Pax5* may still be sufficient for the differentiation process to occur normally. Although I introduced the *Pax5* gRNAs into naïve B cells, there would be a delay between transduction and the loss of *Pax5* in these cells, meaning that in this system *Pax5* is being lost after the cells are activated. My results suggest that, in contrast to the dedifferentiation observed following loss of *Pax5* in naïve B cells, targeting *Pax5* after activation accelerates the rate of ASC differentiation. This increased differentiation is likely due to more a rapid loss of other negative regulators whose expression is driven by *Pax5*, including *Bach2*, however, this remains to be confirmed experimentally. *Sdc1*/CD138 is a

known target of Pax5 repression, therefore, it is possible that targeting *Pax5* is not altering the rate of differentiation, but simply increasing the proportion of CD138⁺ cells (Horcher et al., 2001). Future work will examine the antibody secretion of these CD138⁺ cells to ensure that they are bona fide ASCs.

Interestingly, targeting *Pold1* caused an increase in differentiation to a similar extent as these known key negative regulators. *Pold1* encodes the catalytic subunit of the DNA polymerase delta (PolD) complex, which is involved in the synthesis of the lagging strand during DNA replication and in several DNA damage repair pathways (Nicolas et al., 2016). Given its known roles in DNA replication, it is possible that targeting *Pold1* is affecting B cell proliferation. It is known that B cell division and B cell differentiation are intrinsically linked as the probability that a cell will undergo differentiation increases with each cell division (Hasbold et al., 2004; Hodgkin et al., 1996). Recent work has shown that the rate of B cell differentiation is also influenced by the length of time that it takes the cell to divide, specifically the time spent in the G₁ phase of cell cycle (Chan et al., 2019; Zhou et al., 2018). Treatment of activated B cells with Purvalanol A, a cyclin dependent kinase inhibitor that prolongs the time cells spend in G₁, causes a dramatic increase in the proportion of Blimp-1 positive cells (Chan et al., 2019). Interestingly, several studies have implicated *Pold1* in the transition from G₁ to S-phase. Reduction of *Pold1* protein levels through RNAi reduces the rate of cell cycle progression, particularly the entry into S-phase (Song et al., 2015; Tumini et al., 2016). Two recent studies have also identified *Pold1* mutations in individuals with immunodeficiencies. Mutations that inhibit PolD complex formation or block *Pold1* DNA polymerase activity have both been shown to reduce T cell proliferation and delay the transition from G₁ to S-phase (Conde et al., 2019; Cui et al., 2020). Patients with *Pold1* mutations display a slight reduction in the serum levels of IgA and IgG, however, this decrease has been attributed to T cells being unable to provide help to the B cell compartment rather than a B cell intrinsic defect (Cui et al., 2020). To date, *Pold1* has not been implicated in the proliferation of B cells. I propose targeting *Pold1* in B cells is likely causing a delay in the transition from G₁ to S-phase and that this prolonged time in G₁ resulted in an increased proportion of cells undergoing differentiation.

This screen also identified 7 genes that had a more modest effect on the rate of differentiation. *Arhgef18* is one of 24 known guanine exchange factors (GEFs) that activate RhoA activity and several of these GEFs are expressed in B cells (Bros et al., 2019; Ricker et al., 2016). RhoA is involved in signal transduction following the ligation of the B cell receptor, CD40, Toll-like receptors (TLRs) or chemokine receptors, and has been shown to influence B cell proliferation and migration following activation (Bros et al., 2019; Ricker et al., 2016). RhoA has also been reported to activate Pten activity (Li et al., 2005). Pten inhibits the activity of PI3K and *Pten*^{-/-} B cells display enhanced differentiation (Setz et al., 2018). It is possible that the increase in differentiation following targeting of *Arhgef18* is due to a dysregulation of the RhoA/Pten/PI3K pathway. Interestingly, overexpression of *Arfgef28*, another GEF that activates RhoA activity, increases B cell differentiation (Ha et al., 2012). It is possible that multiple GEFs may be working co-operatively or antagonistically in B cells to modulate RhoA activity and control the rate of differentiation. A role for Rps6ka5, also called Msk1, in regulating LPS-TLR4 signalling has previously been studied in bone marrow-derived macrophages (Ananieva et al., 2008). TLR4 signalling activates the MAPK signalling pathway and leads to Rps6ka5 activation. Rps6ka5 induces expression of *Dusp1*, which dephosphorylates components of the MAPK cascade leading to downregulation of MAPK signalling. To date, it has not been demonstrated if Rps6ka5 plays a similar role in repressing MAPK signalling in B cells, however, the results from my screen suggest that it might. It is possible that targeting Rps6ka5 results in sustained MAPK signalling following LPS stimulation, which leads to increased proliferation and differentiation. The increased differentiation in *Ripk3* targeted cells is surprising as *Ripk3*^{-/-} B cells appear to display normal proliferation and differentiation responses (Newton et al., 2004). At this stage, the reason for this discrepancy is unclear. The remaining genes, *AB124611*, *A430078G23Rik*, *Fam43a* and *Rnf130*, have no known function and, therefore, present intriguing candidates for further investigation to understand how they are influencing differentiation.

This screen also revealed that targeting *Eef1a1*, which encodes a translation elongation factor, results in a complete absence of differentiated cells. Knockdown of *Eef1a1* expression induces cell death, and it is likely that the

absence of differentiated cells following targeting of *Eef1a1* is either due to death of the CD138⁺ cells or death of the B cells before they are able to differentiate (Bao et al., 2020; Dapas et al., 2020). It has also been proposed that knockdown of *Eef1a1* causes a proliferative block, however, these claims have been based on total cell number counts and using this parameter the effects on cell death and cell division cannot be clearly separated (Bao et al., 2020; Dapas et al., 2020). If targeting *Eef1a1* does cause a severe proliferative defect, then cells may not be able to progress through enough divisions to be able to differentiate. Future work investigating the role of *Eef1a1* on B cell differentiation should include analysis of proliferation and survival.

There are multiple genes that have a known role in regulating the rate of differentiation that were not identified as hits by this screening approach. Bcl6, another key negative regulator of B cell differentiation, was included in the arrayed screen, however, it did not increase the rate of differentiation. This result agrees with Bcl6 inhibiting the differentiation of GC B cells into ASC but not the extrafollicular plasmablast response (Dent et al., 1997). Other known negative regulators *Id3* and *Irf8*, were not included in the investigated gene set as they did not reach the expression fold-change cut-off that I used in the criteria for inclusion in my gene set. It is likely that there are genes that have a role in inhibiting differentiation were excluded from the gene set that I interrogated, however, as discussed previously, a balance must be reached between increasing the number of investigated genes and the practicality of performing an arrayed screen on primary cells.

It should also be noted that the functions of some genes may not be evident when using a knockout screening approach due to redundancy. A clear example of this is *Spib*, a gene with a known role in repressing B cell differentiation that was not identified as a hit in this screen. *Spib*^{-/-} B cells only display a slight increase in the rate of differentiation due to partial redundancy between SpiB and PU.1/*Spi1* (Willis et al., 2017). It is likely that there are other negative regulators included in my gene set that were not identified as hits due to redundancy and using a knockout screening approach may not be suitable to identify these genes. An alternate method would be to perform a complementary screen that utilizes

modified Cas9 proteins. For example, using dCas9, which maintains expression of target genes, may reveal a role in inhibiting differentiation that is not resolvable in a knockout screen (Gilbert et al., 2014; Konermann et al., 2015). While *Irf8*, *Spib* or *Spi1* deficient B cells only display slight alterations in their rates of differentiation, ectopic expression of *SpiB*, *Irf8* or *Spi1* results in a clear block in differentiation (Carotta et al., 2014; Schmidlin et al., 2008; Willis et al., 2017). This suggests that a dCas9 mediated screen would be able to identify these genes whose involvement in repressing differentiation is not evident in the knockout screen that I have performed. These dCas9 screens would build upon the work that I have presented here to identify additional negative regulators and generate a more complete understanding of how the B cell differentiation process is controlled.

**Chapter 5. Irf4 is
essential for B-1a cell
development through
the activation of the
transcription factor
Bhlhe41**

5.1. Introduction

B1 cells are a subpopulation of B murine lymphocytes that are considered to be more innate-like than conventional B2 cells. They play an important role in the early immune response as they are the producers of “natural” antibodies, which are antibodies secreted without prior antigen encounter, while B2 cells are predominantly responsible for the antigen-driven immune response (Hayakawa et al., 1983; Savage and Baumgarth, 2015). Functionally, B1 cells display a more rapid response to stimulation than B2 cells, which has led to the view that they are poised for the early response to pathogen encounter (Fairfax et al., 2007). B1 cells also show a decreased propensity to undergo class-switch recombination (CSR) to IgG isotypes (Kitabatake et al., 2015; Tarlinton et al., 1995). B1 cells are distinguishable from B2 cells by both their location and surface marker expression. While B2 cells are the predominant B lymphocyte populations in the spleen and lymph nodes, B1 cells are rare in secondary lymphoid organs but form the majority of the B cell population within the peritoneal cavity (Hayakawa et al., 1985; Hayakawa et al., 1983). In contrast to peritoneal B2 cells (B220⁺CD23⁺IgD^{hi}IgM^{lo}), B1 cells are identified by the surface phenotype of B220^{+/lo}CD23⁻IgD^{lo}IgM^{hi}. Additionally, B1 cells can be further segregated based on CD5 expression, with CD5⁺ B1 cells being called B-1a cells, and CD5⁻ B1 cells are called B-1b cells (Stall et al., 1992).

Although B-1a and B-1b cells are similar in many respects, each subset plays a non-redundant role within the humoral immune response (Smith and Baumgarth, 2019). It is thought that B-1a cells are the primary producers of poly-reactive IgM which is secreted in the absence of antigen exposure and has a protective role in the early response to Influenza and *Streptococcus pneumoniae* responses (Baumgarth et al., 1999; Haas et al., 2005). In contrast, B-1b cells are thought to respond in an antigen-specific manner and form immunological memory, although there is some evidence that B-1b cells may also be capable of spontaneous antibody secretion (Alugupalli et al., 2004; Haas et al., 2005; Savage and Baumgarth, 2015). In addition to this, B-1a cells have a restricted B cell receptor (BCR) repertoire and a proportion of B-1a cells express

BCRs that recognise self-antigens (Hayakawa et al., 1990; Hayakawa et al., 1984; Pennell et al., 1989a).

The existence of B1 cells in humans remains a topic of debate within the field. CD5⁺ human B cells were initially thought to be a homologous population to mouse B-1a cells, however, subsequent studies revealed that CD5 can also be expressed in transitional and activated B2 cells in humans (Kageyama and Katayama, 2020). In mice, the majority of B1 cells are located within the peritoneal and pleural cavities, however, sample collection from these sites in humans is difficult. A population of human B cells present in cord blood and adult peripheral blood has been demonstrated to spontaneously secrete antibody *in vitro*, raising the possibility that these may be equivalent to mouse B1 cells, however, whether this population has a similar function to mouse B1 cells *in vivo* remains to be determined (Griffin et al., 2011; Rothstein et al., 2013).

In contrast to B2 cells, which differentiate from hematopoietic stem cells (HSCs) in the bone marrow, B1 cells are predominantly derived from foetal and neonatal HSCs and are maintained in adult mice through a process of self-renewal (Hayakawa et al., 1985; Hayakawa et al., 1986; Kantor et al., 1992; Montecino-Rodriguez et al., 2016). There are currently two B1 precursor populations that have been identified, B1 progenitors (Lin⁻CD93⁺CD19⁺B220^{lo}), which are capable of developing into both B-1a and B-1b cells, and B-1a transitional cells (CD93⁺IgM⁺CD19⁺B220^{lo}CD5⁺), which can only become B-1a cells (Montecino-Rodriguez et al., 2006; Pedersen et al., 2014). BCR signalling has a critical role in B-1a cell development. Transgenic B cells that express the B-1a restricted BCRs Vh12/Vk4 or Vh12/Vk9 will preferentially develop into B-1a cells (Arnold et al., 1994; Chumley et al., 2000). Furthermore, a recent study has shown that expression of B-1a restricted BCRs in mature B2 cells will result in B2 cells differentiating into cells that phenotypically and transcriptionally resemble bona fide B1 cells (Graf et al., 2019). Mutations that alter BCR signalling strength will influence the development of B1 cells. Loss of function mutations in genes that positively regulate BCR signalling result in a reduction in the peritoneal B-1a population (Kreslavsky et al., 2018). Conversely, mice carrying genetic alterations that increase BCR signal strength, either through deletion of negative

regulators or overexpression of positive regulators, have an expanded B-1a compartment.

Although B1 and B2 cells share many aspects of their transcriptional program, a small number of transcriptional regulators and signalling molecules have been shown to have distinct roles in the development and function of B1 cells. *Pou2f2/Oct2* deficient mice have a complete absence of the peritoneal B1 population (Humbert and Corcoran, 1997). *Arid3a*^{-/-} mice have an almost complete absence of peritoneal B-1a cells, while *Arid3a* transgenic mice have expanded peritoneal B-1a and splenic marginal zone B cell compartments (Hayakawa et al., 2019). *Bhlhe41* and *Nfkbid/IkBNS* are both required for B-1a cell development to progress past the transitional B-1a stage (Kreslavsky et al., 2017; Pedersen et al., 2014). It is likely, however, that there are additional factors that are required for the generation of the peritoneal B1 populations.

Irf4 is a transcription factor that has important roles in many immune cell subsets and its role in the development and function of B2 cells has been studied extensively (De Silva et al., 2012; Nam and Lim, 2016). *Irf4* has important roles in the development of B2 cells, where it functions redundantly with *Irf8* to downregulate the pre-BCR and allow development to progress past the pre-B cell stage (Lu et al., 2003). In mature B2 cells, *Irf4* is essential for the formation of germinal centres, CSR and the differentiation into antibody secreting cells (ASCs) (Klein et al., 2006; Mittrücker et al., 1997; Ochiai et al., 2013; Sciammas et al., 2006; Willis et al., 2014). Interestingly, *Irf4* appears to have concentration dependent functions as low expression of *Irf4* in activated B2 cells is important for CSR, while high *Irf4* levels drive ASC differentiation (Sciammas et al., 2006). The importance of *Irf4* in the development and function of B1 cells, however, remains unclear.

Here I have found an essential role for *Irf4* in B-1a cells, as *Irf4* deficient (*Irf4*^{-/-}) mice are lacking in this population. I have shown that the absence of B-1a cells is due to a defect in their development at the B-1a transitional cell stage. Transcriptional analysis of *Irf4*^{-/-} B1 cells revealed a role for *Irf4* in promoting the expression of *Bhlhe41*, which has recently been identified as a transcription factor

that is essential for the development and function of B-1a cells (Kreslavsky et al., 2017).

5.2. Results

5.2.1. *Irf4*^{-/-} mice lack peritoneal B-1a cells

Previous studies investigating the steady state lymphocyte numbers of *Irf4*^{-/-} mice have described dramatic alterations in their B cell populations, most strikingly, a complete absence of ASCs (Klein et al., 2006; Mittrücker et al., 1997; Ochiai et al., 2013; Sciammas et al., 2006; Willis et al., 2014), however the role of *Irf4* within the more innate-like B1 cell populations remains unclear. To investigate a potential role of *Irf4* in B1 cells, I compared the B lymphocyte populations in the spleen, BM and peritoneal cavity of *Irf4*^{-/-} and WT mice. In agreement with previous work, *Irf4*^{-/-} mice had a reduction in the frequency, number and phenotype of follicular B cells, an expansion of marginal zone B cells in the spleen when compared with WT mice, and an absence of plasma cells in the spleen and BM (Mittrücker et al., 1997) (**Figure 5.1**). *Irf4*^{-/-} and WT mice had no difference in the overall frequency of B1 cells within the peritoneal cavity (**Figure 5.2a,b**). However, subdividing the B1 population into CD5⁺ B-1a and CD5⁻ B-1b cells revealed a dramatic reduction the frequency of B-1a cells in *Irf4* deficient mice when compared to WT mice (**Figure 5.2c,d**). Curiously, the B-1b population was still present in the *Irf4*^{-/-} mice. To investigate whether the difference in *Irf4* dependency between B-1a and B-1b cells was due to a difference in *Irf4* expression, I compared *Irf4* expression in B cell populations using an *Irf4*-GFP reporter mouse. *Irf4*-GFP expression was detectable in all B cell populations, with the lowest expression in splenic follicular B (FoB) cells and the highest expression in plasma cells (**Figure 5.2e**). Peritoneal B-1a cells displayed a slightly higher expression of *Irf4*-GFP compared to the peritoneal B-1b population. Together these data show that the expression of *Irf4* is essential for the generation and/or survival of B-1a cells, but not B-1b cells.

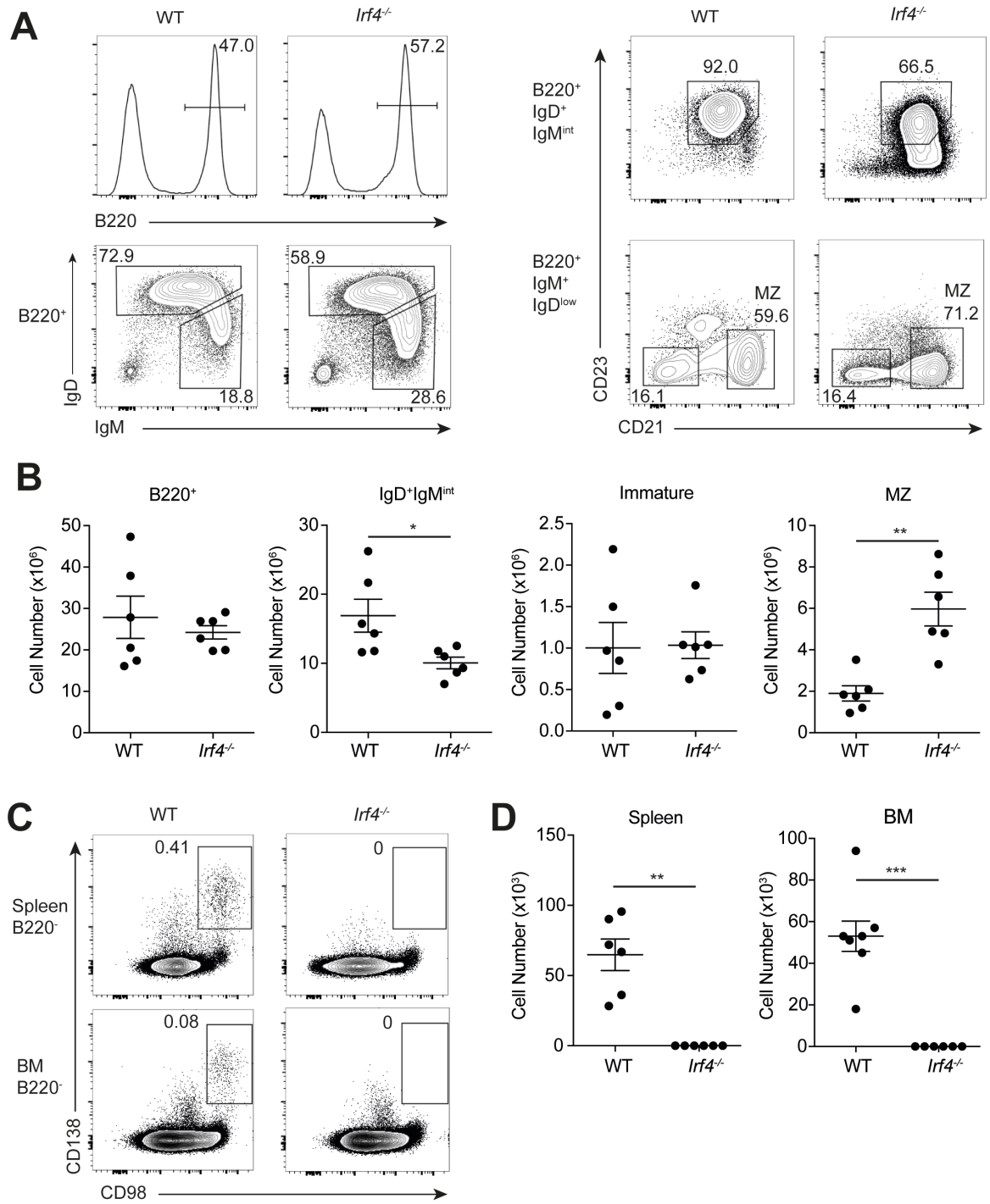


Figure 5.1 *Irf4*^{-/-} mice have alterations in spleen and bone marrow B lymphocyte populations.

(A) Representative flow cytometry gating of B220⁺ splenocytes, B220⁺IgD⁺IgM^{int}, Immature and MZ B cells in the spleen of *Irf4*^{-/-} and age-matched WT mice. (B) Total number of B220⁺ splenocytes, B220⁺IgD⁺IgM^{int}, Immature and MZ B cells in the spleen of *Irf4*^{-/-} and age-matched WT mice. (C) Representative flow cytometry gating of B220⁻CD98^{hi}CD138⁺ plasma cells in the spleen and bone marrow of *Irf4*^{-/-} and age-matched WT mice. (D) Total number of plasma cells in the spleen and bone marrow of *Irf4*^{-/-} and age-matched WT mice. Data are combined from 2 independent experiments. Statistical significance was determined using an unpaired t-test. Error bars represent the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

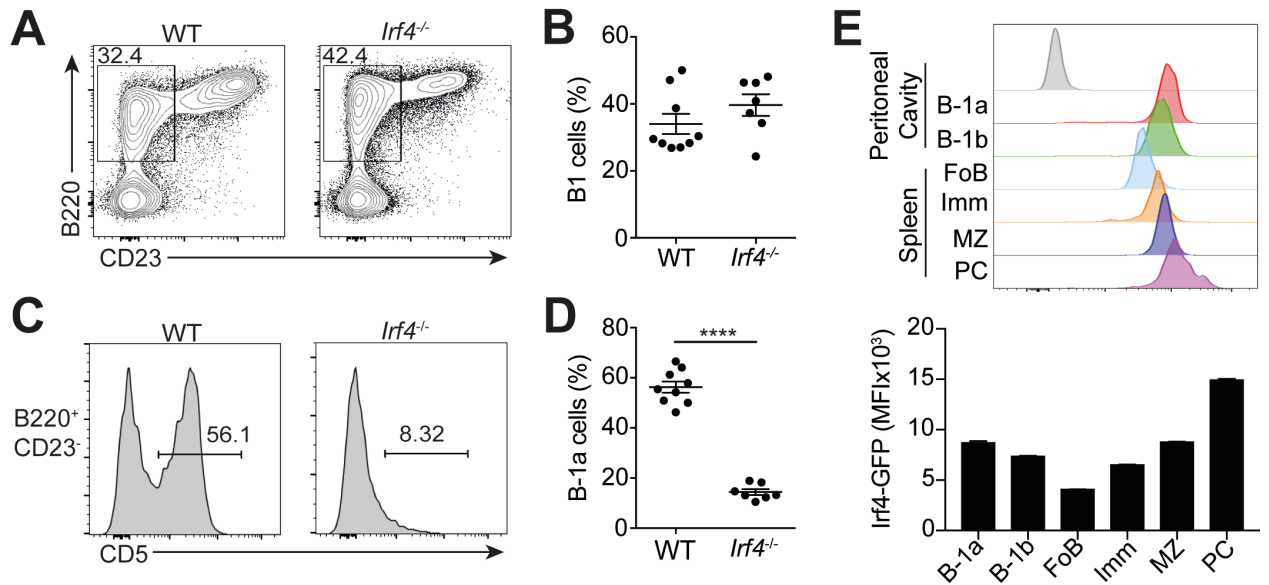


Figure 5.2 *Lrf4*^{-/-} mice lack peritoneal B-1a cells.

(A) Representative flow cytometry plots for identifying B1 cells in the peritoneal cavity of *Lrf4*^{-/-} and WT mice. Numbers indicate the percentage of cells within the gate. (B) Frequency of B1 cells among peritoneal cells from *Lrf4*^{-/-} and age-matched WT mice. Each symbol represents an individual mouse. (C) Representative histograms showing the proportion of peritoneal B1 cells that are CD5 positive in *Lrf4*^{-/-} and WT mice. (D) Proportion CD5⁺ cells among peritoneal B1 cells (CD23-B220^{int}) in *Lrf4*^{-/-} and aged-matched WT mice. Data are combined from 3 independent experiments where *n* = 3-4. Error bars represent the mean ± SEM. (E) Representative histograms showing the expression of *Lrf4*-GFP in the indicated B lymphocyte populations from the spleen and peritoneal cavity of *Lrf4*-GFP reporter mice. The average MFI of GFP in each population is shown below. Error bars represent the SD. *n* = 2.

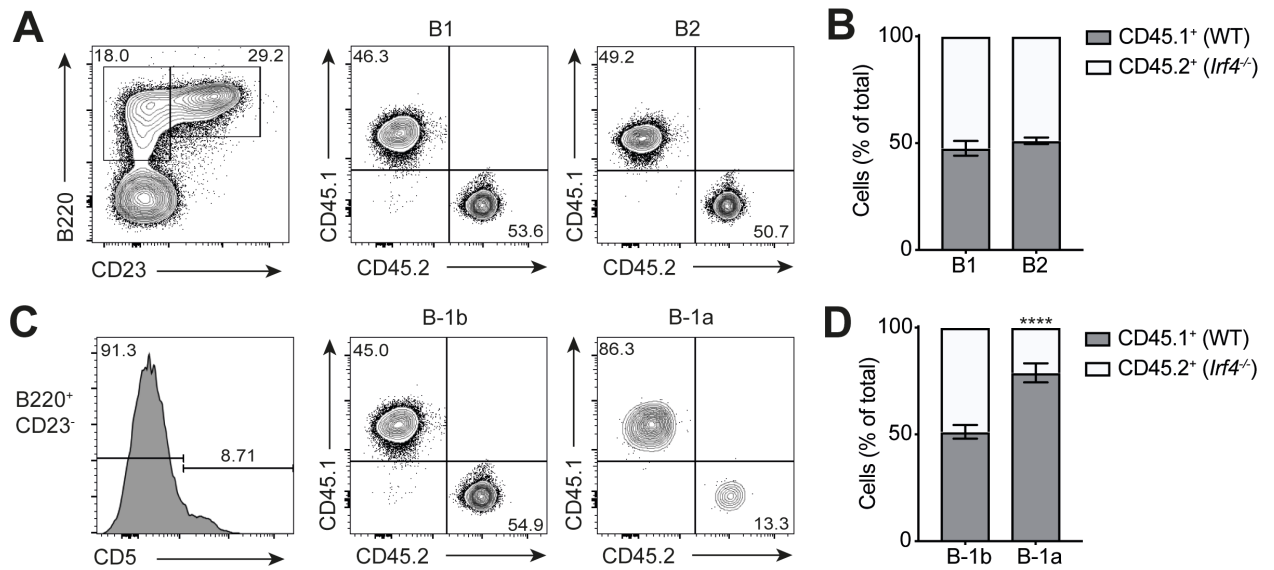


Figure 5.3 The loss of the B-1a population in *Irf4*^{-/-} mice is cell intrinsic.

Mixed chimeras were generated by transferring a 1:1 mixture of WT (CD45.1⁺) and *Irf4*^{-/-} (CD45.2⁺) E14.5 foetal liver cells to lethally irradiated CD45.1⁺CD45.2⁺ recipient mice. (A) Representative flow cytometry plots showing gating of CD45.1⁺ and CD45.2⁺ cells within peritoneal B cell subsets in chimera mice. (B) Frequency of CD45.1⁺ (WT) and CD45.2⁺ (*Irf4*^{-/-}) cells among peritoneal B1 (CD23⁻B220^{int}) and B2 (CD23⁺B220^{hi}) cells. (C) Representative flow cytometry plots showing gating of CD45.1⁺ and CD45.2⁺ cells within peritoneal B1 cell subsets in chimera mice. (D) Frequency of CD45.1⁺ (WT) and CD45.2⁺ (*Irf4*^{-/-}) cells among peritoneal B-1a (CD23⁻B220^{int}CD5⁺) and B-1b (CD23⁺B220^{hi}CD5⁺) cells. Data are representative of 2 independent experiments of *n* = 6. Statistical significance was determined using an unpaired *t*-test. Error bars represent the mean ± SEM. **** *p* < 0.0001.

5.2.2. The absence of B-1a cells in *Irf4*^{-/-} mice is cell intrinsic

To determine whether the absence of B-1a cells in *Irf4*^{-/-} mice was due to a cell intrinsic defect mixed-foetal-liver chimeras were generated by transferring equal numbers of foetal liver (FL) cells from WT (CD45.1⁺) and *Irf4*^{-/-} (CD45.2⁺) E14.5 mouse embryos into lethally irradiated mice (CD45.1⁺CD45.2⁺). After waiting 8 weeks for reconstitution, the peritoneal B cell populations were analysed, and the relative contribution of each donor cell genotype was compared. There was an equal contribution of WT and *Irf4*^{-/-} donor cells to the peritoneal B2 and total B1 compartments (**Figure 5.3a,b**). There was also an equal contribution of WT and *Irf4*^{-/-} donor cells to the B-1b population, however, B-1a cells were predominantly derived from the WT donor cells (**Figure 5.3c,d**). This indicated that the B-1a cell deficiency that I observed in the *Irf4*^{-/-} mice was the result of a cell intrinsic defect.

5.2.3. *Irf4* is required for B-1a cell development

B-1a cells are predominantly generated during foetal and neonatal development and are maintained in adult mice through self-renewal. During their development, B-1a cells progress through two distinct stages, B1 progenitor cells which are committed to becoming B1 cells but retain the capacity to differentiate into B-1a or B-1b cells, and B-1a transitional cells which are committed to becoming B-1a cells (Montecino-Rodriguez et al., 2006; Pedersen et al., 2014). To investigate whether *Irf4* has a role in the development of B-1a cells, I examined the B1 progenitor cell population (BCR^{lin}-CD93⁺CD19⁺B220^{int}) in neonatal *Irf4*^{-/-} mice. There was a slight, but non-significant reduction in the frequency of B-1 progenitors in the spleen of neonatal *Irf4*^{-/-} mice when compared to age-matched WT mice (**Figure 5.4a,b**). B1 progenitors are also present in the BM of adult mice, however, this population is transcriptionally distinct from neonate B1 progenitors (Montecino-Rodriguez et al., 2016). I examined the B1 progenitor population within the BM of adult *Irf4*^{-/-} mice to determine whether the absence of *Irf4* has a different effect on this population. There was no significant difference in the frequency of B-1 progenitor cells in the BM of adult *Irf4*^{-/-} mice when compared to age-matched WT mice (**Figure 5.4a,b**). I next examined the effect of *Irf4* deficiency on B-1a transitional (Tr-B1a) cells (CD93⁺IgM⁺CD19⁺B220^{int}CD5⁺). Tr-B1a cells were largely absent from the

spleens of 10 day old *Irf4*^{-/-} pups (**Figure 5.4c,d**). This suggests that *Irf4* is required for either the generation or maintenance of this transitional population and that the absence of B-1a cells in adult *Irf4*^{-/-} mice is likely the result of a defect in B-1a cell development at the B-1a transitional stage.

5.2.4. CD5⁻ *Irf4*^{-/-} B1 cells are true B-1b cells

From my data it is evident that *Irf4* is essential for the generation of peritoneal B-1a cells, however, it is unclear why their sister population, B-1b cells, appear to be unaffected by the absence of *Irf4*. There is still little that is understood about how the B-1a and B-1b populations differ and there are no clues in the current literature as to why these populations would be differentially affected by *Irf4* function. To determine how B-1a and B-1b cells differ transcriptionally, and how the loss of *Irf4* alters this transcriptional profile, RNA-sequencing analysis was performed on splenic follicular B cells, peritoneal B-1a and B-1b cells from WT mice, and splenic follicular B cells and the CD5⁻ B1 population from *Irf4*^{-/-} mice.

As the strategy used only distinguished the peritoneal B-1a and B-1b populations by the surface expression of CD5 and the identification of the Tr-B1a population is dependent on CD5 expression, I needed to eliminate the possibility that *Irf4* was regulating *Cd5* expression. If B-1a cells were present in the *Irf4*^{-/-} mice but had lost expression of CD5, then the CD5⁻ population should contain a mixture of B-1a and B-1b cells. As shown by principal component analysis, the CD5⁻ B1 cells from the *Irf4*^{-/-} mice clustered closely with WT B-1b cells, and away from the WT B-1a and splenic follicular B cells, indicating that these *Irf4*^{-/-} CD5⁻ B1 cells are true B-1b cells and not B-1a cells with an altered surface marker phenotype (**Figure 5.5**).

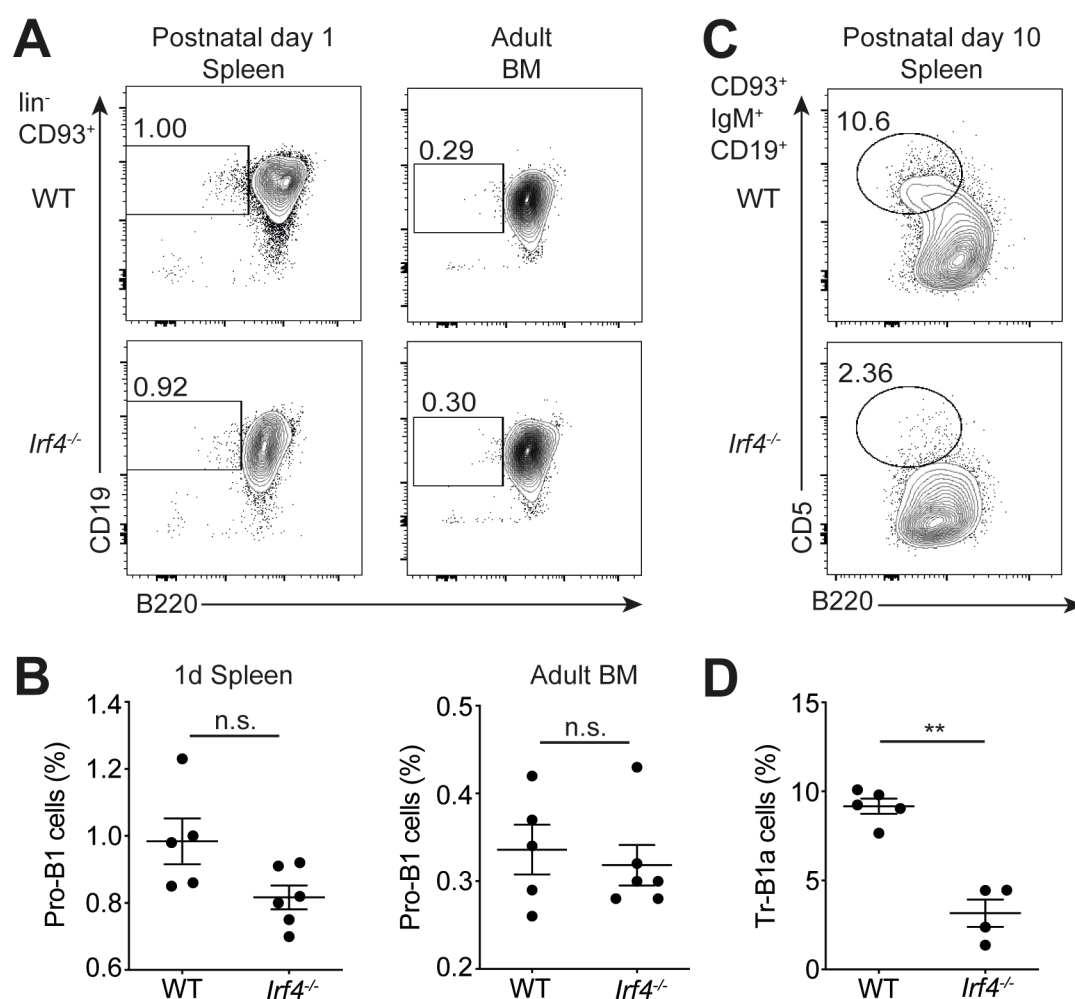


Figure 5.4 B-1a transitional cells are absent in *Irf4*^{-/-} mice.

(A) Representative flow cytometry gating for the identification of B1 progenitor (pro-B1) cells in the spleen of 1 day old and the BM of adult *Irf4*^{-/-} and WT mice. (B) Frequency of Pro-B1 cells among lin⁻CD93⁺ cells in the spleen of 1 day old and the BM of adult *Irf4*^{-/-} and WT mice. (C) Representative flow cytometry gating for the identification of B-1a transitional (Tr-B1a) cells in the spleen of 10 day old *Irf4*^{-/-} and WT mice. (D) Frequency of B-1a transitional cells among CD93⁺IgM⁺CD19⁺ cells in the spleen of 10 day old *Irf4*^{-/-} and WT mice. Data are combined from 2 independent experiments of *n* = 2-3. Statistical significance was determined using an unpaired *t*-test. Error bars represent the mean ± SEM. ** *p* < 0.01.

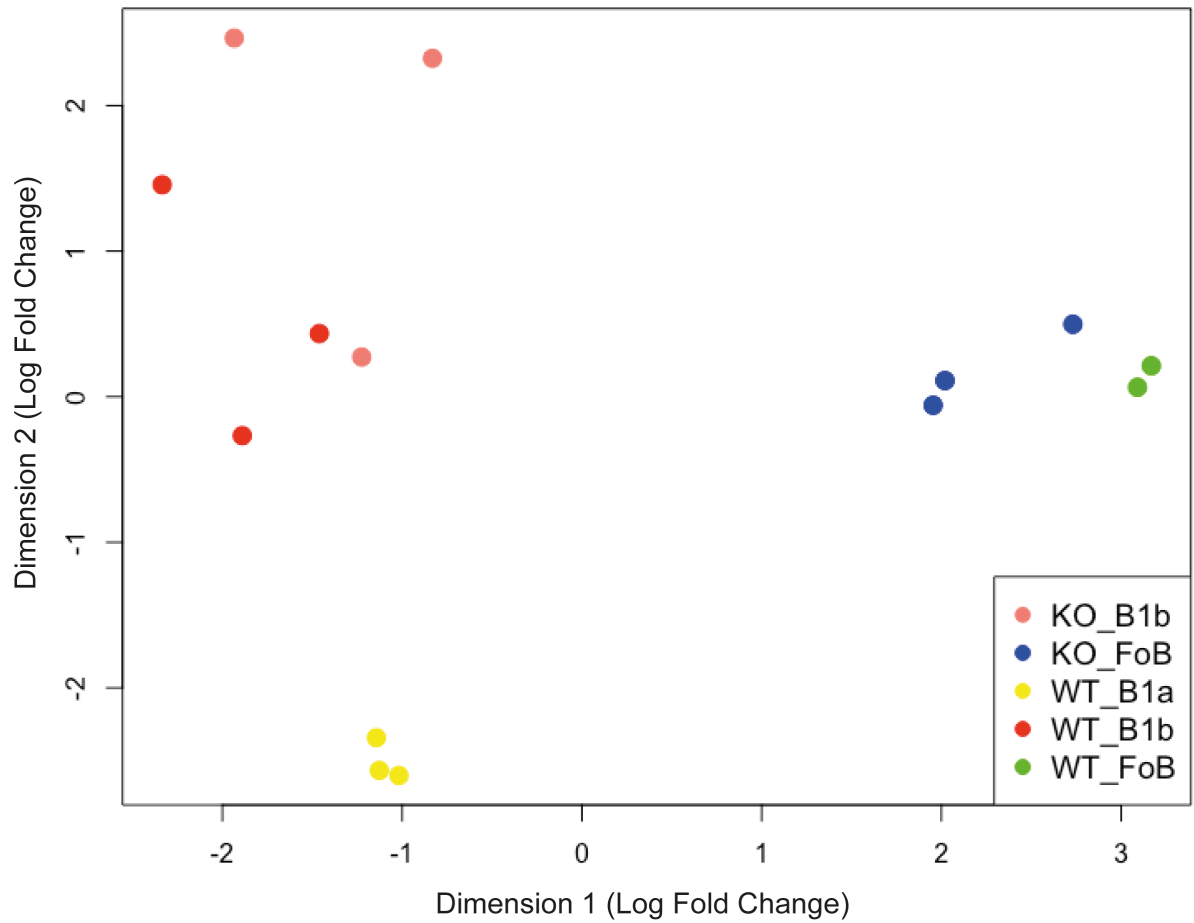


Figure 5.5 MDS clustering of WT peritoneal B-1a, B-1b and splenic FoB cells with *Irf4*^{-/-} peritoneal B1 cells.

The top 1000 differentially expressed genes between each pair of samples were selected for clustering. Each dot represents a single sample, coloured coded for identity and genotype as indicated.

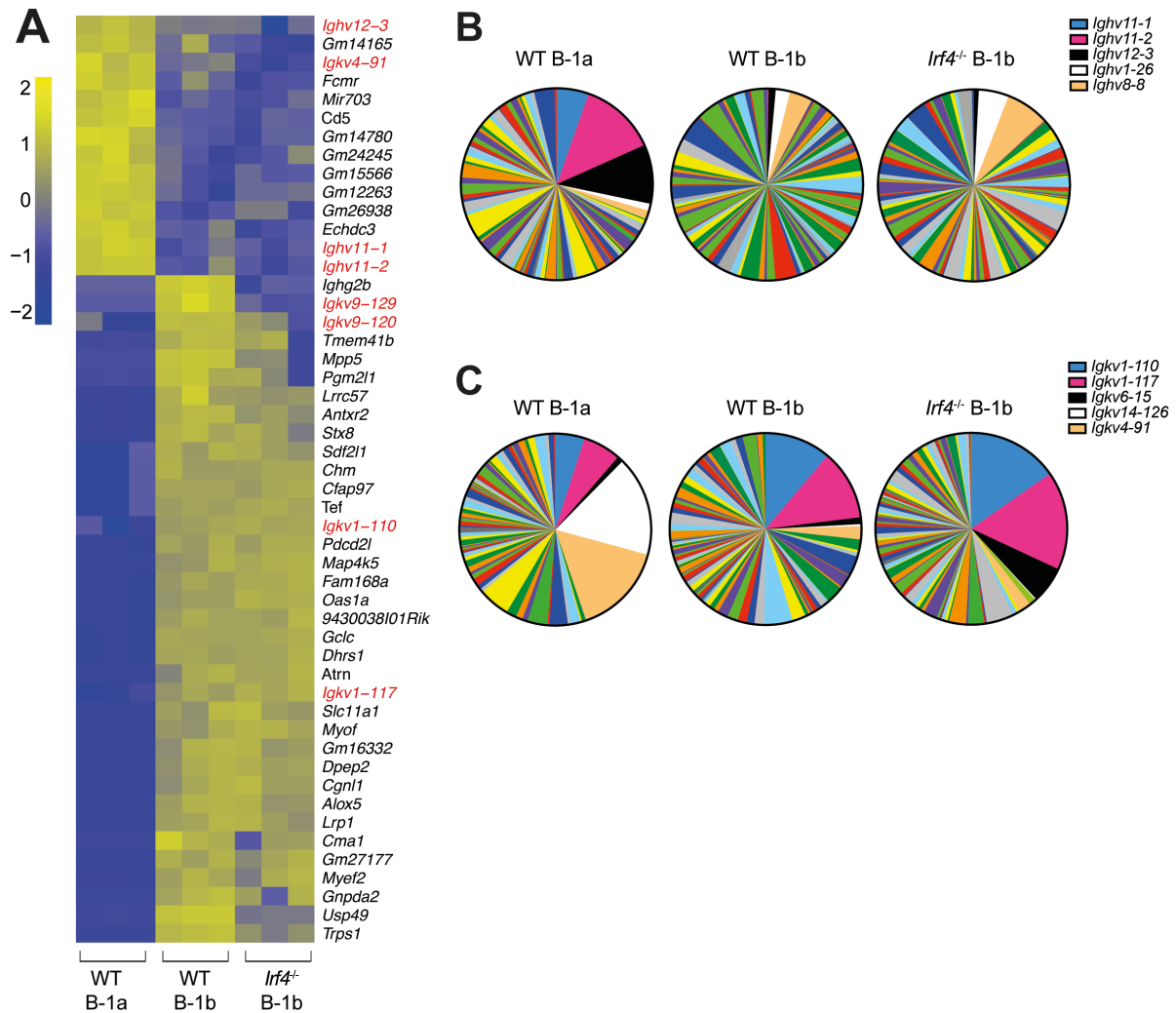


Figure 5.6 Transcriptional profiling of WT and *Irf4*^{-/-} peritoneal B1 cells.

RNA-sequencing was performed on CD5⁺ and CD5⁻ B1 cells sorted from the peritoneal of WT and *Irf4*^{-/-} mice. (A) Expression profile of the top 50 differentially expressed genes (DEGs) between WT B-1a and WT B-1b cells (adjusted *p*-value ≤ 0.05 , fold-change ≥ 1). *Ighv* and *Igkv* genes are indicated in red. Each replicate sample contains cells pooled from 3 age- and sex-matched mice. Heatmap is colour-coded according to Z score. (B) Fraction of reads that mapped to each *Ighv* gene out of the total reads that mapped to an *Ighv* gene for each population. Data are presented as the average fraction across replicates for each cell type. (C) Fraction of reads that mapped to each *Igkv* gene out of the total reads that mapped to an *Ighv* gene for each population. Data are presented as the average fraction across replicates for each cell type.

Next, I identified the differentially expressed genes (DEGs) between WT B-1a and WT B-1b (Adjusted p-value ≤ 0.05 , average $\log_2$ expression ≥ 1 , expression $\log_2$ fold change ≥ 1) (**Figure 5.6a**). This analysis only revealed 107 DEGs (92 with higher expression in B-1b and 15 with higher expression in B-1a), suggesting that peritoneal B-1a and B-1b cells are transcriptionally very similar. Many of the genes that were differentially expressed between these two populations encoded variable (V) regions from immunoglobulin genes. B-1a cells are known to have a restricted BCR repertoire, with large proportion of B-1a cells expressing Vh12Vk4 or Vh11Vk14 (Pennell et al., 1989a). This bias towards *Ighv11* and *Ighv12* usage by WT B-1a cells was clearly evident in my analysis (**Figure 5.6b**). In contrast, WT B-1b cells did not show this pattern of *Ighv* usage. WT B-1b cells displayed a bias towards usage of *Igkv1-110* and *Igkv1-117*, while *Igkv14-126* and *Igkv4-91*, the predominate V-regions used by B-1a cells, only constituted a small fraction of total *Igkv* usage (**Figure 5.6c**). To increase confidence that the CD5⁺ B1 cells from *Irf4*^{-/-} mice were true B-1b cells, I examined the *Ighv* and *Igkv* usage in this population. The *Ighv* usage pattern of *Irf4*^{-/-} CD5⁺ B1 cells closely matched WT B-1b cells as they did not display a bias towards *Ighv11* and *Ighv12*. The *Igkv* usage of this population also matched the *Igkv* pattern of WT B-1b cells as there was a clear enrichment for usage of *Igkv1-110* and *Igkv1-117*, and a very small contribution of *Igkv14-126* and *Igkv4-91*. Together, the V-region usage patterns and comparison of the transcriptional profiles of these populations strongly suggest that Tr-B1a and B-1a cells are absent in *Irf4*^{-/-} mice and have not simply lost their expression of CD5.

Next, I utilised gene ontology analysis to perform an unbiased comparison of the gene expression profiles of the WT B-1a and WT B-1b populations. As there were few DEGs between these populations, there were no pathways that were identified as being significantly enriched in either the B-1a or B-1b population. I reanalysed the data with no average expression cut-off to increase the number of genes that would be included (115 genes with higher expression in B-1b, 52 with higher expression in B-1a). Despite removing the expression cut-off, this analysis revealed few differences between the two populations, and these differences were largely driven by the presence of *Ighv* and *Igkv* gene segments

in the DEG lists (**Figure 5.7**). From these data, I conclude that there are few transcriptional differences between resting peritoneal WT B-1a and B-1b cells.

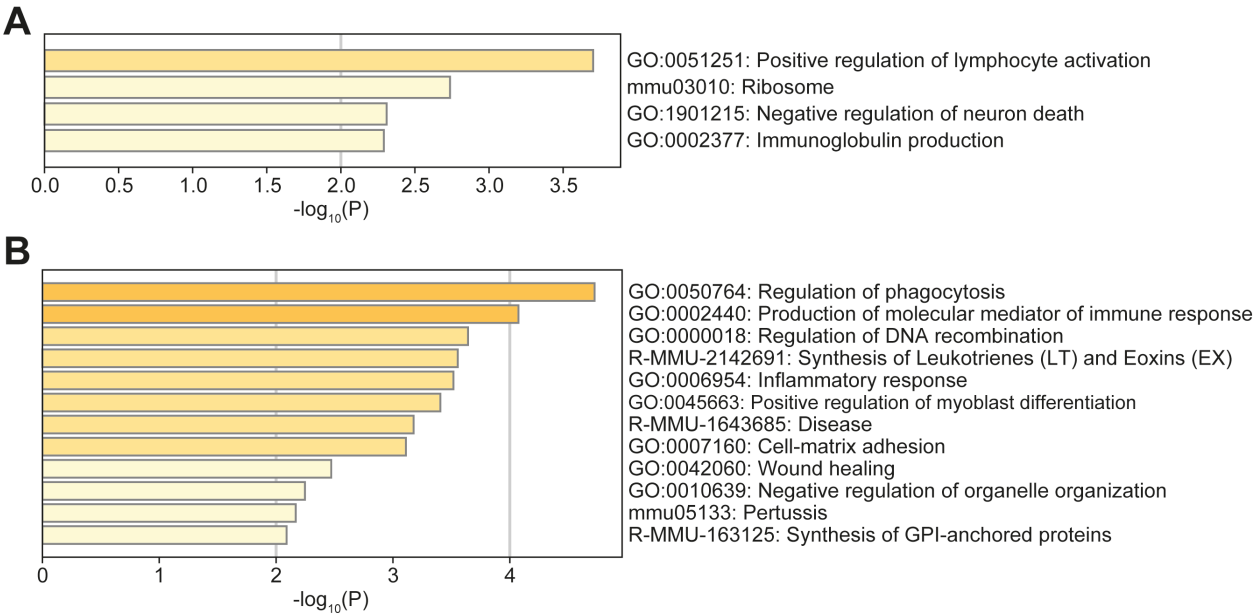


Figure 5.7 Few gene pathways are differentially expressed between B-1a and B-1b cells.

*RNA-sequencing analysis was performed as described in **Figure 5.6**. (A) Gene ontology analysis on genes upregulated in WT B-1a cells compared to WT B-1b cells. (B) Gene ontology analysis on genes upregulated in WT B-1b cells compared to WT B-1a cells.*

5.2.5. *Irf4* regulates expression of *Bhlhe41*, a transcription factor that is essential for B-1a development and function

To investigate how *Irf4* was influencing the biology of B1 cells, I compared the transcriptional profiles of WT and *Irf4*^{-/-} peritoneal B-1b cells. This analysis identified 38 DEGs between the two populations with 25 genes being upregulated in the *Irf4*^{-/-} B-1b cells and 13 genes being downregulated (Adjusted p-value ≤ 0.05 , average log₂ expression ≥ 1 , expression log₂ fold change ≥ 1) (**Figure 5.8a**). In agreement with the data from the *Irf4*-GFP reporter mice (**Figure 5.2e**), *Irf4* expression was detected in both the B-1a and B-1b population (**Figure 5.8b**). The expression of *Irf8* was increased in *Irf4*^{-/-} B-1b cells compared to WT B-1b cells, which agrees with previous reports demonstrating that *Irf8* is upregulated in *Irf4* deficient B cells (Xu et al., 2015).

To determine whether the expression of any genes previously implicated in B1 cell development were being affected by the absence of *Irf4*, I examine the changes in gene expression of *Arid3a*, *Pou2f2*, *Nfkbid*, *Cd19* and *Bhlhe41* (**Figure 5.8b**). Of these known regulators, only the expression of *Pou2f2* and *Bhlhe41* was significantly affected by the absence of *Irf4*. *Pou2f2*, which encodes Oct2, is involved in B1 cell development and *Pou2f2*^{-/-} mice lack both B-1a and B-1b cells (Humbert and Corcoran, 1997). However, as expression of *Pou2f2* is increased in the *Irf4*^{-/-} B-1b cells, it is unlikely that aberrant *Pou2f2* expression is responsible for the phenotype that I observe in the *Irf4*^{-/-} mice. In contrast to this, expression of *Bhlhe41* expression was abolished in the absence of *Irf4*. *Bhlhe41* is a transcription factor that has been shown to be essential for peritoneal B-1a cell development and function, and the alteration in *Bhlhe41* expression that I observed in the *Irf4*^{-/-} B1 cells was particularly interesting as the B1 cell phenotype of the *Irf4*^{-/-} mice closely matches with the phenotype observed in *Bhlhe41*^{-/-} mice (Kreslavsky et al., 2017). *Irf4*^{-/-} B-1b cells displayed a slight but non-significant increase in expression of *Bhlhe40*, a close homolog of *Bhlhe41* that has been proposed to have a redundant function in some cell types (Rauschmeier et al., 2019).

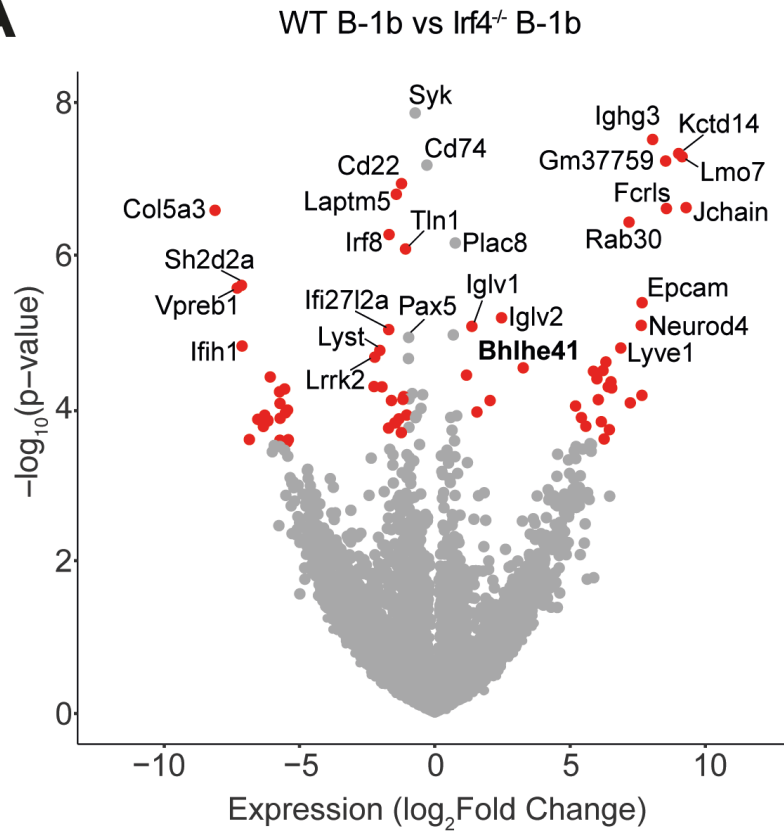
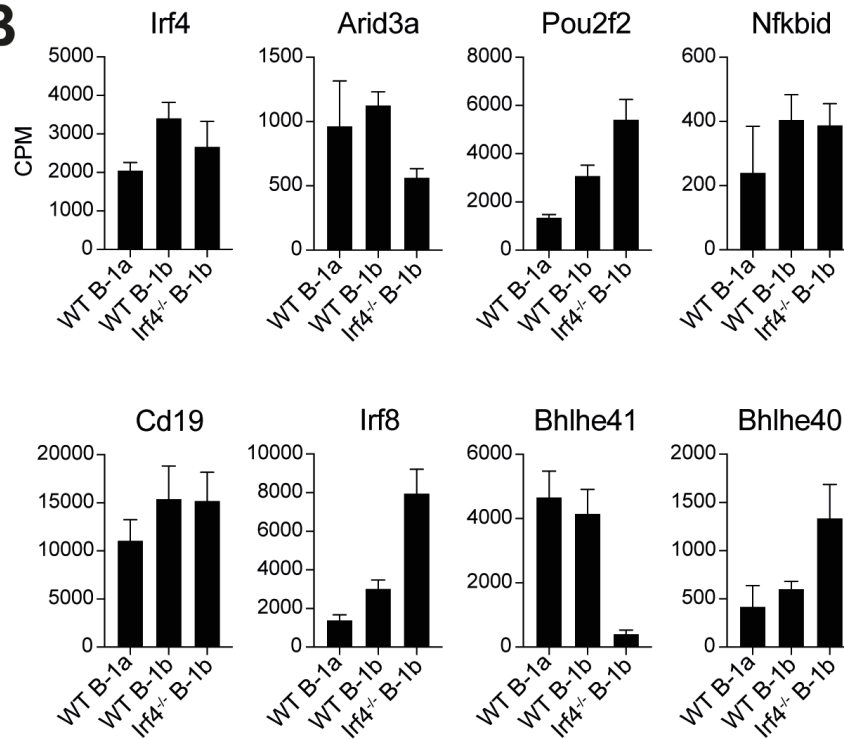
A**B**

Figure 5.8 Transcriptional analysis of *Irf4*^{-/-} peritoneal B-1b cells.

RNA-sequencing analysis was performed as described in **Figure 5.6**.

(A) Volcano plot showing DEGs between WT and *Irf4*^{-/-} B-1b cells. Red dots indicate significant DEGs (adjusted *p*-value ≤ 0.05 , average expression ≥ 1 , fold change ≥ 1). (B) Average counts per million (CPM) of *Irf4*, *Irf8*, *Arid3a*, *Pou2f2*, *Nfkbid*, *Cd19*, *Bhlhe40* and *Bhlhe41*.

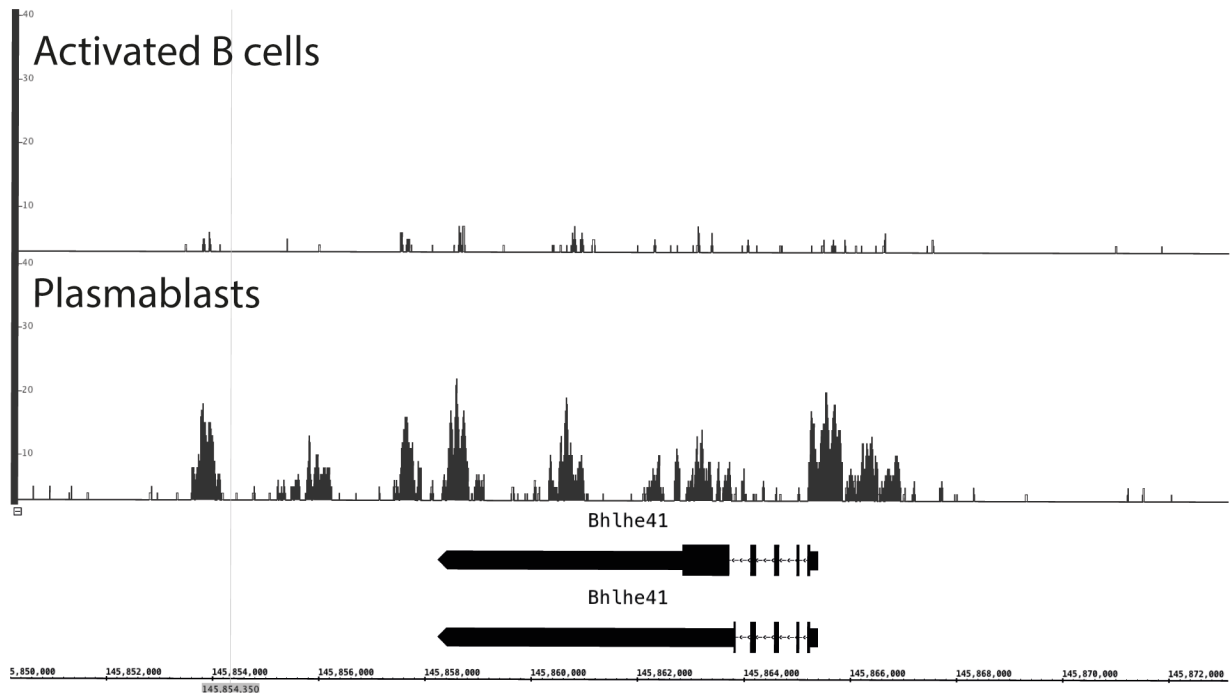


Figure 5.9 *Irf4* binds to the *Bhlhe41* locus

Irf4 ChIP-sequencing data showing *Irf4* binding around *Bhlhe41* gene locus in LPS stimulated B cells and plasmablasts (*Blimp-1*⁺*CD138*⁺). Error bars represent mean $\pm$ SEM. Data were obtained from Minnich et al, Nature Immunology (2016).

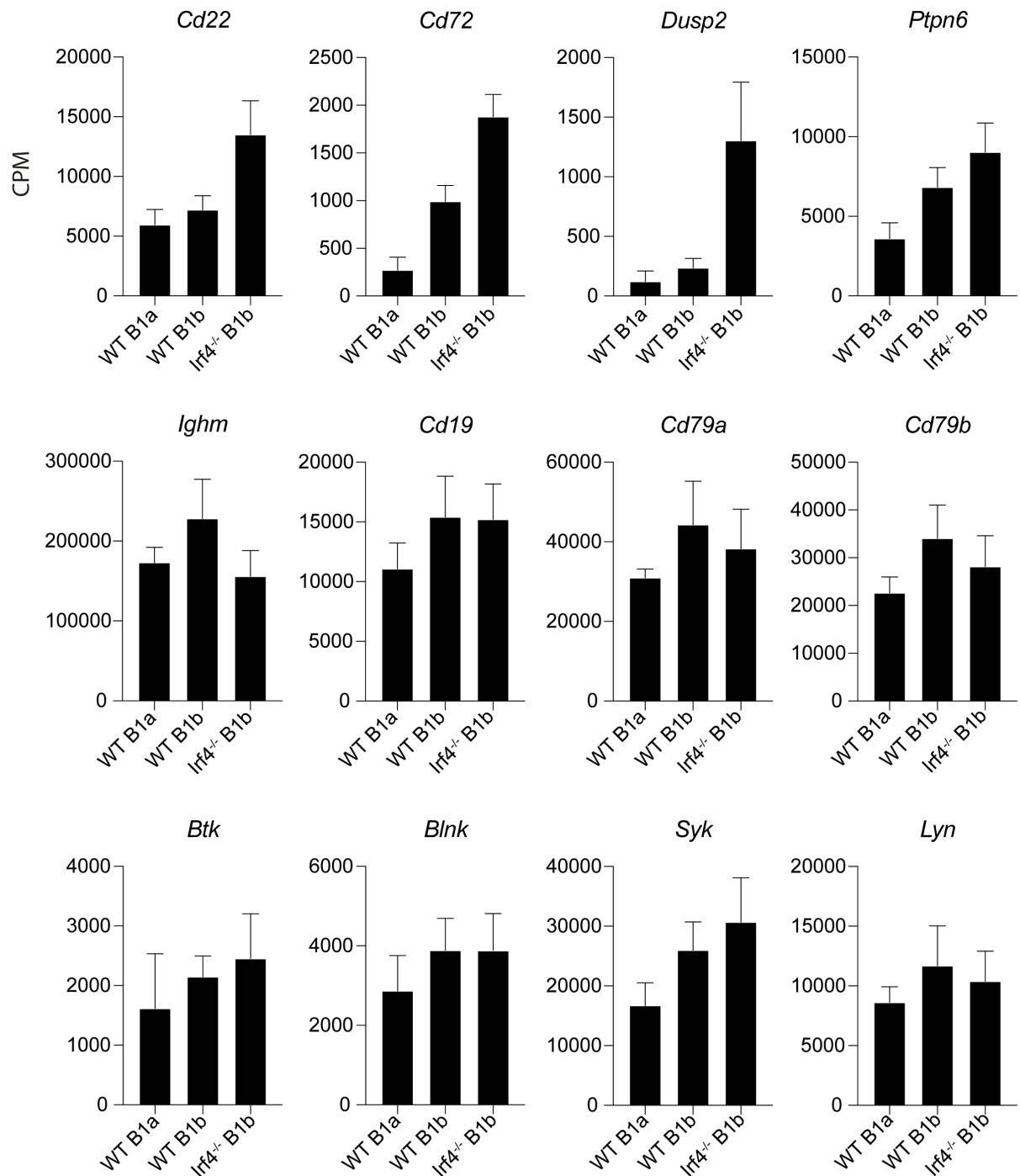


Figure 5.10 *If4^{-/-}* B-1b cells have altered expression of BCR signalling regulators. RNA-seq analysis was performed as described in **Figure 5.6**. Expression of various regulators of BCR signalling are shown. CPM, counts per million. Error bars represent the mean ± SEM.

To further investigate a potential role for *Irf4* in the activation of *Bhlhe41* expression, I interrogated ChIP-seq data previously published by Minnich et al, which examined *Irf4* binding in LPS activated B cells and plasmablasts (Minnich et al., 2016). This analysis showed clear binding of *Irf4* to the *Bhlhe41* locus, which suggests that *Irf4* may directly induce *Bhlhe41* expression (**Figure 5.9**).

5.2.6. *Irf4* deficient B1 cells have altered BCR signaling and proliferation

Among the genes that were significantly upregulated in the *Irf4*^{-/-} B-1b cells was *Cd22*, which encodes a negative regulator of BCR signalling. This observation was intriguing as *Bhlhe41* has been proposed to be required for B-1a cell proliferation and homeostasis through its direct repression of negative regulators of BCR signalling (Kreslavsky et al., 2017). To further investigate the relationship between *Irf4* and *Bhlhe41* in B1 cells, I examined the changes in expression of other genes involved in BCR signalling between WT and *Irf4*^{-/-} B-1b cells. I did not observe any significant changes in the expression of positive regulators of BCR signalling (*Ighm*, *Cd19*, *Cd79a*, *Cd79b*, *Btk*, *Syk* or *Blnk*) (**Figure 5.10**). In contrast to this, the expression of *Cd72* and *Dusp2*, negative regulators of BCR signalling that are directly repressed by *Bhlhe41*, was increased (Kreslavsky et al., 2017). Similar to what was observed in *Bhlhe41*^{-/-} B1 cells, *Irf4*^{-/-} cells had a slight but non-significant increase in expression of *Ptpn6*/SHP-1 and no alterations in the expression of *Lyn*. Together, these expression patterns add further support to the notion that *Irf4* regulates *Bhlhe41*.

I next wanted to do an unbiased analysis of the transcriptional differences between WT and *Irf4*^{-/-} B1 cells so I performed gene set enrichment analysis (GSEA). GSEA revealed significant increase in expression of E2F transcription factors target genes (**Figure 5.11**). E2F transcription factors drive the expression of genes required for cell cycle progression (Thurlings and de Bruin, 2016). GSEA also revealed an upregulation of genes involved in G₂-M cell division checkpoint progression in *Irf4*^{-/-} B-1b cells. (**Figure 5.11**). Interestingly, the derepression of cell cycle genes, including E2F target genes, was also observed in *Bhlhe41*^{-/-} B1 cells (Kreslavsky et al., 2017). Together, these data point to a role for *Irf4* in

regulating the generation and homeostasis of peritoneal B-1a cells through the direct activation of *Bhlhe41* expression.

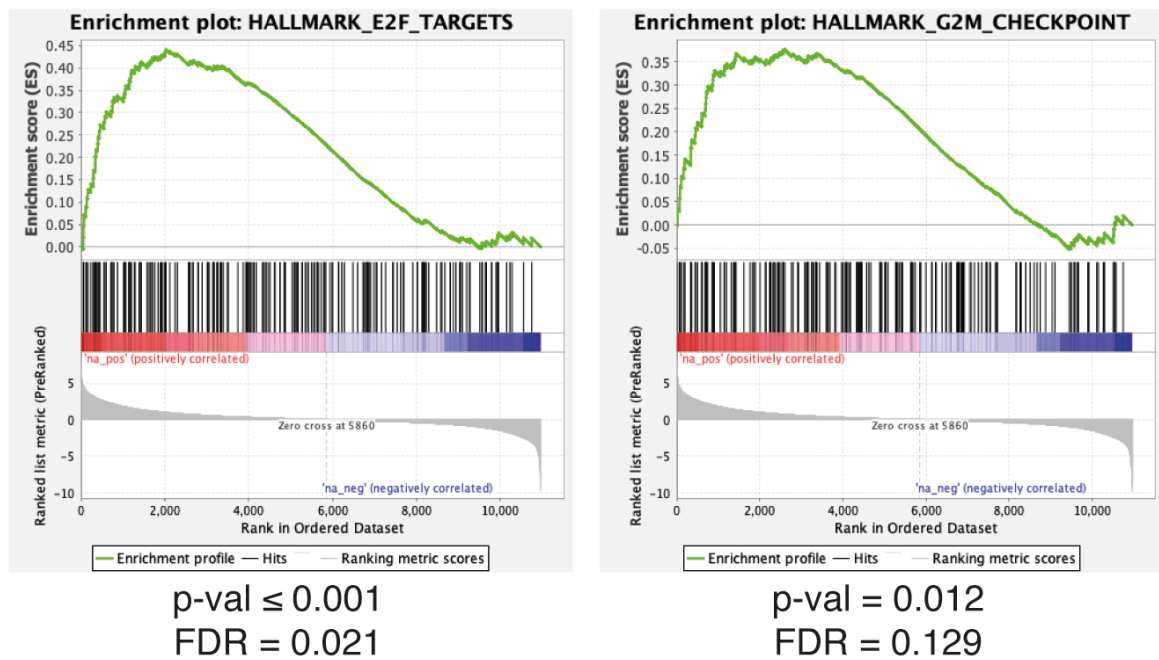


Figure 5.11 *Irf4*^{-/-} B-1b cells have increased expression of cell cycle genes. RNA-seq analysis was performed as described in **Figure 5.6**. GSEA of E2F target genes (Molecular signatures database: HALLMARK_E2F_TARGETS) and G₂-M checkpoint genes (Molecular signatures database: HALLMARK_G2M_CHECKPOINT) in *Irf4*^{-/-} B-1b cells relative to WT B-1b cells.

5.3. Discussion

Previous studies have demonstrated that *Irf4* is an important transcription factor that is indispensable for many processes within the B lymphocyte lineage, however, its role in the more innate-like B1 cell populations remains poorly understood. Here I have demonstrated a novel role for *Irf4* in the generation of the peritoneal B-1a cell population. I have shown that *Irf4*^{-/-} mice have a block in B-1a cell development at the earliest known stage of B-1a cell commitment, transitional B-1a cells. Consistent with my finding that this transitional population was dependent on *Irf4*, the mature B-1a population was almost entirely absent from *Irf4*^{-/-} mice.

Curiously, the peritoneal B-1b population appeared to be indifferent to the absence of *Irf4*. One potential explanation for this discrepancy could be that only the B-1a population expresses *Irf4*, however, using an *Irf4*-GFP reporter mouse I have demonstrated that both B-1a and B-1b cells express similar levels of *Irf4* (**Figure 5.2e**). Another potential explanation could be that *Irf4* is not expressed during B1 cell development until the B-1a committed Tr-B1a stage. In support of this notion, progenitor B1 cells, which are still capable of differentiating into either B-1a or B-1b cells, were present at similar numbers and frequencies between *Irf4*^{-/-} and WT neonates (**Figure 5.4**). However, previous work has shown that *Irf4* is expressed in the earlier progenitor B1 population, which can still develop into B-1a or B-1b cells (Montecino-Rodriguez et al., 2016). B-1a cells are predominantly generated from foetal liver progenitors and are maintained in adult mice through self-renewal, while B-1b cells are able to be generated from both foetal liver and adult bone marrow progenitors (Kantor et al., 1992). It is possible that only foetal and neonatal B1 cell development may be affected by that absence of *Irf4*, however, my data does not support this theory. In competitive mixed-foetal-liver reconstitutions, the *Irf4*^{-/-} donor cells repopulated the peritoneal B-1b compartment just as efficiently as the WT donor cells (**Figure 5.3**), suggesting that foetal B1 cell development is not impaired and *Irf4* does not play a role B-1b cell generation. As the identification of both B-1a and Tr-B1a cells are dependent on expression of CD5, it is possible that the perceived loss of these populations in the *Irf4*^{-/-} mice is merely the result of a loss of CD5 expression. By comparing the transcriptomes and Ig V-region usage patterns of

WT B-1a, WT B-1b and *Irf4*^{-/-} CD5⁻ B1 cells (**Figures 5.5 and 5.6**), I have demonstrated that the CD5⁻ B1 population in the *Irf4*^{-/-} mice solely consist of B-1b cells and, therefore, the B-1a population is truly absent in these mice.

Having eliminated these possible explanations for the differential dependency of B-1a and B-1b cells on *Irf4*, I sought to understand the differences between these two populations using RNA-seq analysis. While the differences between B-1a and B-1b cells remains poorly understood, it is thought that both populations have distinct roles within the immune system, with B-1a cells spontaneously secreting natural antibodies and B-1b cells producing an antigen-specific response forming a long-term immunological memory (Haas et al., 2005; Smith and Baumgarth, 2019). Surprisingly, I identified very few transcriptional differences between the peritoneal B-1a and B-1b populations, and many of the differentially expressed genes were mapped to Ig V-regions. An unbiased analysis of changes in pathway and gene sets failed to reveal any clear differences between these two populations, therefore, I concluded that there are few transcriptional differences between B-1a and B-1b cells, at least in their resting state. It is possible that these populations have post-translational differences which would not be identified by my RNA-seq analysis and would require a proteomics-based approach to understand. Alternatively, these differences in B-1a and B-1b may not be evident until after activation. A key difference between B-1a and B-1b cells is the expression of CD5. A previous study has shown that CD5 acts as a negative regulator of BCR signalling that inhibits B-1a cells from proliferating following stimulation with anti-IgM and pushes them towards apoptosis (Bikah et al., 1996). Therefore, it is possible that the differences between B-1a and B-1b cells may only become apparent after activation.

Previous studies have identified several factors that regulate the development of either the total B1 cell pool or specifically B-1a cells, including *Arid3a*, *Pou2f2/Oct2*, *CD19*, *Nfkbid/1κBNS* and *Bhlhe41* (Engel et al., 1995; Hayakawa et al., 2019; Humbert and Corcoran, 1997; Kreslavsky et al., 2017; Pedersen et al., 2014; Rickert et al., 1995; Sato et al., 1996b). My RNA-seq analysis revealed an ablation of *Bhlhe41* expression in the absence of *Irf4*. *Bhlhe41* has been

reported to be essential for the generation and homeostasis of peritoneal B-1a cells, while being dispensable for the development of the B-1b population (Kreslavsky et al., 2017). I have multiple threads of evidence to suggest that the absence of the peritoneal B-1a population that I observe in the *Irf4*^{-/-} mice is likely the result of *Irf4* directly inducing the expression of *Bhlhe41*. Firstly, I have shown that *Bhlhe41* expression is almost completely absent in the *Irf4*^{-/-} B-1b cells (**Figure 5.8**). Analysis of previously published *Irf4* ChIP-seq data shows clear binding of *Irf4* at the *Bhlhe41* locus (Minnich et al., 2016). Secondly, the phenotype of the peritoneal B1 compartment in *Irf4*^{-/-} mice closely mirrors that of *Bhlhe40*^{-/-} *Bhlhe41*^{-/-} mice (Kreslavsky et al., 2017). Both mutant strains show block in B-1a development at the transitional B-1a stage resulting in an absence of peritoneal B-1a cells, while the B-1b populations appear normal. Finally, there are shared changes in gene expression patterns between WT and *Irf4*^{-/-} B1 cells, and between WT and *Bhlhe40*^{-/-} *Bhlhe41*^{-/-} B1 cells. *Bhlhe41* has been proposed to be required for peritoneal B-1a cell generation and homeostasis through its direct repression of negative regulators of BCR signalling (*Cd72* and *Dusp2*) and cell cycle regulators (*Cdkl1*, *Ccnh*, *Cks2*, *E2f2*, *E2f7* and *E2f8*) (Kreslavsky et al., 2017). Similarly, my RNA-seq analysis of *Irf4*^{-/-} B-1b cells shows that there is a derepression of negative regulators of BCR signalling and of cell cycle regulators (**Figures 5.10 and 5.11**). Together, these data suggest that the B-1a phenotype observed in the *Irf4*^{-/-} mice is due to a lack of *Bhlhe41* expression. To test this hypothesis, *Bhlhe41* could be ectopically expressed in *Irf4*^{-/-} B1 progenitor cells. If the expression of *Bhlhe41* in these progenitor cells is sufficient to restore the peritoneal B-1a population, this supports a model where the direct activation of *Bhlhe41* expression by *Irf4* promotes B-1a cell development. However, this still does not explain why B-1a appear to be selectively sensitive to loss of *Irf4*. Similar to *Irf4*, *Bhlhe41* is expressed at similar levels in both B-1a and B-1b cells and, therefore, the specific loss of the B-1a population cannot be explained by a difference in expression.

Strong BCR signalling is required for B-1a development and mice carrying mutations in BCR signalling or co-stimulatory molecules often have altered B-1a populations (Berland and Wortis, 2002; Kreslavsky et al., 2018). Mice deficient in positive regulators of BCR signalling, CD19 (Engel et al., 1995; Rickert et al.,

1995), Btk (Khan et al., 1995), CD21/35 (Ahearn et al., 1996), Vav-1 (Tarakhovsky et al., 1995; Zhang et al., 1995), BLNK (Pappu et al., 1999), have a reduction in their peritoneal B-1a population. Conversely, mice that are deficient in negative regulators, CD72 (Pan et al., 1999), Lyn (Chan et al., 1997), CD22 (O'Keefe et al., 1996; Sato et al., 1996a), have an expansion of B-1a cells. My RNA-seq analysis revealed an increase in the expression of negative regulators of BCR signalling in *Irf4*^{-/-} B1 cells. A potential explanation for the absence of B-1a cells is that this increase in expression of negative regulators results in reduced signalling through the BCR during B1 cell development, and consequentially these cells would not receive sufficiently strong BCR signalling to become B-1a cells.

These results build upon previous work to understand the genetic regulation of B-1a cell development. I have revealed a novel role for the transcription factor *Irf4* in promoting the generation of peritoneal B-1a cells. These data suggest that *Irf4* acts upstream of *Bhlhe41*, which has been previously demonstrated to be required for B-1a cell development (Kreslavsky et al., 2017). I propose that in the absence of *Irf4*, *Bhlhe41* is not expressed, resulting in a developmental block in B-1a cell development at the transitional B-1a stage.

Chapter 6. Future directions and conclusions

The humoral immune response constitutes a critical part of the adaptive immune response to infection. Much work has been done over the past decades to understand the underlying genetic networks controlling how B cell subsets develop and how these cells then differentiate into ASCs. Although many genetic factors have been revealed to be involved in the generation of B cells and ASCs, we still lack a complete understanding of how the ASC differentiation process is controlled and how the biology of ASCs is regulated. The work presented in this thesis aims to address these issues using a series of genetic perturbation screens to identify novel genetic regulators of ASC differentiation and function.

6.1. Future work

The major aim of this thesis was to develop and perform a series of CRISPR/Cas9 mediated genetic screens on primary mouse B cells to identify novel regulators of the humoral immune response. Through these screens I have identified a number of novel regulators that impact on proliferation, survival, differentiation and antibody secretion *in vitro* in response to the T cell independent stimulus LPS, and these results highlight many avenues for future investigations. Many of the genes identified in my screen have not been previously associated with B cell or ASC biology and some of these genes do not have a known function, therefore, further investigation into these novel regulators is required to determine the mechanism behind their effects. Future work is required to determine whether these novel regulators are also required for these processes in response to conditions that mimic T cell dependent stimulation. The defective CD40L response that I have observed in the Cas9 expressing B cells (**Figure 3.4**) means that the systems that I have developed in this thesis will not be a suitable approach to investigate this question. One potential alternate method to identify regulators important for the CD40L response would be to use an RNA interference screen to knockdown expression of target genes. This does not result in the same complete loss of gene function that using CRISPR/Cas9 achieves, but this can be performed using WT B cells that will mount an appropriate response to CD40L stimulation. It also remains to be established whether these genes have a similar role in the B cell response *in vivo*. While the

in vitro system that I have used provides a highly sensitive method to identify genes involved in B cell differentiation, it does not fully recapitulate the *in vivo* generation of ASCs and it is possible that these genes may not have the same effects in both settings (Shi et al., 2015). Furthermore, the majority of genes within the ASC gene signature did not measurably influence any of the parameters that I have measured. It is possible that additional genes within the ASC gene signature will be required for the differentiation process *in vivo*, or for other processes such as homing to the plasma cell niche or long-term survival. Finally, it would be interesting to determine if these novel regulators have a conserved function in the human humoral response. Identification of genes that regulate ASC differentiation, survival or antibody secretion can represent potential therapeutic avenues for the manipulation of these processes in vaccine responses or diseases.

One possible application of the screening technique that I have developed is in the identification of genes involved in immune disorders, such common variable immunodeficiency (CVID). CVID is a heterogenous clinical classification and patients are characterized by having low serum levels of IgG and IgA, and mount poor humoral responses to vaccination (Ghafoor and Joseph, 2020). As a consequence, CVID patients suffer from recurrent infections throughout their life and are typically treated with monthly immunoglobulin infusions. The underlying genetic causes of CVID can be mono- or polygenic, and the genetic cause for the majority of CVID patients is unknown. Advances in genomic technologies allowing whole exome sequencing of CVID patients has resulted in increased identification of disease-causing mutations, with an estimated discovery rate of 25-60% of patients having mutations in genes with known functions (Kienzler et al., 2017). However, for the remaining CVID patients, the challenge remains in determining which polymorphisms are having a deleterious effect on the B cell response. I believe that the arrayed screening approach in primary B cells that I have developed will be useful system to investigate this question. This arrayed screening approach presents a highly sensitive method to identify genes that have a cell-intrinsic effect on B cell differentiation, survival, proliferation and antibody secretion. The arrayed screen for positive regulators of B cell differentiation identified *Sec61a1* as a gene that is essential for the generation of

ASCs. Recently, loss-of-function mutations in *Sec61a1* have been identified as the disease causing mutations in 2 separate families with CVID, and B cells isolated from these patients display impaired ASC differentiation (Schubert et al., 2018). This suggests that this assay will be suitable for determining which polymorphisms detected in CVID patients are responsible for disrupting the B cell response. These screens also revealed a role for 35 genes in the antibody secretion process, many of which have no previous association with the secretion process. If the function of these genes are conserved in humans, it is possible that CVID patients may harbour mutations in these genes. Therefore, it may be beneficial to consider polymorphisms in these novel regulators of secretion as potential disease-causing mutations. Additionally, this screening method can be easily modified to investigate potential defects in class-switching either through flow cytometric analysis or ELISAs for non-IgM isotypes. This approach could also be complimented with a activating dCas9-based screen as some polymorphisms could result in constitutive activation of the gene product and this effect may not be resolvable in a knockout screen (Gilbert et al., 2014; Konermann et al., 2015).

6.2. Potential implications

Advances in our knowledge of how ASC differentiation and function are regulated will have implications on many aspects of human health. The generation of ASCs is important for responses to pathogens and immunisation, however, inappropriate ASC responses can result in pathologies such as autoimmune conditions, immunodeficiencies and cancer. The generation of long-lived ASCs is essential for sustained protective immunological memory following infection or immunization, therefore, a more complete understanding of the positive and negative regulators of ASC differentiation may be able to inform vaccine design. The goal of almost all vaccine strategies is to generate a population of long-lived plasma cells that will produce high-affinity protective antibodies for our entire life-span. However, we still don't have a clear understanding of how to best engineer vaccines to achieve this. There is significant variation in the length of protective antibody responses generated by current vaccine strategies, with

some vaccines such as smallpox providing life-long protection, while protective anti-tetanus responses decline after several years (Amanna et al., 2007). Different methods of B cell stimulation generate different lifespans of responses, with T cell independent responses typically only lasting for weeks or months, while antigens that cross-link the BCR and elicit T cell help will typically induce an antibody response that can last for decades (Amanna and Slifka, 2010). However, this is not always the case as current influenza vaccines fail to generate long-lived bone marrow plasma cells (Davis et al., 2020). To generate a long-lived plasma cell response a careful balance must be maintained between driving the generation of ASCs and maintaining B cell fate for sufficient time for germinal centre formation to occur. Understanding how the expression of the genes that I have identified are regulated and integrating these novel genes into the current model of how B cell fate decisions are made may assist in the design of high efficacy vaccines.

Specifically targeting the long-lived ASC population in ASC driven autoimmune diseases or in the ASC malignancy multiple myeloma remains challenging. While there are currently effective therapies to specifically deplete the B cell population, such as the anti-CD20 monoclonal antibody Rituximab, these therapies are unable to target the ASC compartment as these surface markers are downregulated during ASC differentiation (Bergantini et al., 2020). The development of monoclonal antibodies targeting multiple myeloma surface proteins CD38 (Daratumumab and Isatuximab) and Slamf7 (Elotuzumab) have improved the treatment of multiple myeloma as they induce the killing of myeloma cells through antibody-dependent cell-mediated cytotoxicity (Storti et al., 2020; Wudhikarn et al., 2020). CD38 and Slamf7 are also expressed on plasma cells and could potentially be used to target the plasma cell population in autoimmune conditions. However, CD38 and Slamf7 expression is not restricted to ASCs and myeloma cells as CD38 is expressed on red blood cells T regulatory cells and myeloid derived suppressor cells, while Slamf7 is expressed on the surface of natural killer cells, DCs and CD8⁺ T cells (Storti et al., 2020; Wudhikarn et al., 2020). Multiple therapies targeting Tnfrsf17/BCMA, which is selectively expressed on the surface of ASCs, are currently in clinical trials, and if approved these therapies may allow for the specific targeting of multiple myeloma cells and

the plasma cell population (Yu et al., 2020). Proteasome inhibitors that can deplete the ASC population, such as Bortezomib, are not truly ASC specific and treatment can result in adverse effects including neuropathy and cardiovascular toxicity (Pancheri et al., 2020). Furthermore, there are currently no approaches to modulate ASC generation or antibody secretion. The genes identified within this thesis may represent novel ways to target the long-lived ASC population.

Mutations in genes that have a known role in either positively or negatively regulating B cell differentiation are frequently associated development and survival of B lineage derived cancers. Inappropriate expression of negative regulators can prevent cells from differentiating and keeps cells in an activated state. For example, dysregulated expression of *Ets1*, *Pax5* and *Bcl6* have all been associated with the development of B cell lymphomas (Garrett-Sinha, 2013; Hatzi and Melnick, 2014; Medvedovic et al., 2011). Similarly, loss of function mutations in the positive regulator of differentiation *Prdm1* are also associated with B cell lymphoma development (Mandelbaum et al., 2010; Xia et al., 2017). Additionally, *Irf4* has been implicated in regulating the survival and proliferation of multiple myeloma cells and targeting *Irf4* in myeloma cells leads to a block in proliferation and rapid cell death (Morelli et al., 2015; Shaffer et al., 2008). It is possible that the novel regulators of B cell proliferation and differentiation that I have identified may also have a role in B lineage derived cancers, and therefore, may highlight novel pathways to target these malignant cells.

One of the most intriguing results from these screens is the identification of many genes that appear to influence antibody secretion without having a measurable effect on ASC differentiation or survival. Future work is required to determine whether these genes are selectively involved in the secretion of antibody or whether they form part of a global secretion pathway. While it is likely that some of the genes identified will fall into the latter category, the identification of truly antibody-specific secretion regulators has clear therapeutic potential. Selectively blocking antibody secretion without affecting ASC survival could be used to augment treatment responses in certain cancer settings while leaving the patients immunological memory intact. Tumour-specific IgG1 antibodies can have strong anti-tumour effects as they can drive complement activation, phagocytosis and

antibody-directed cell cytotoxicity, and the presence of tumour-specific IgG1 is commonly associated with good clinical outcomes in many cancer types (Sharonov et al., 2020). The presence of isotypes other than IgG1, however, is frequently correlated with poor patient outcomes. High levels of serum IgG4 is associated with poor prognosis in melanoma patients and IgG4 antibodies have been shown to interfere with the anti-tumour effects of tumour-specific IgG1 in metastatic melanoma models (Karagiannis et al., 2013). One potential explanation for this correlation that has been proposed is that they may be occupying the Fc receptors on NK cells and macrophages, preventing interaction with tumour-specific antibodies bound to the surface of tumour cells (Sharonov et al., 2020). If endogenous antibody production could be blocked in these patients, it might increase the effectiveness of treatment with monoclonal antibodies directed against tumour cells. Furthermore, high levels of tumour-specific IgA is a poor prognostic marker in many cancer settings and mice that are unable to class-switch to IgA have enhanced responses to chemotherapy in a metastatic prostate cancer model (Shalapour et al., 2015; Sharonov et al., 2020; Welinder et al., 2016). In these settings it may be advantageous to temporarily inhibit antibody production to increase the effectiveness of the treatment.

It is possible that blocking antibody secretion may have detrimental effects on ASC survival *in vivo* that are not detectable in these *in vitro* assays due to the short life span of *in vitro* generated plasmablasts. Increased antibody accumulating within the cell may upset the cellular protein homeostasis and trigger the terminal UPR pathways leading to cell death. Future work should focus on understanding the impact of blocking antibody secretion through the targeting of these genes *in vivo*. If blocking antibody secretion does lead to cell death, then targeting regulators of antibody secretion represents a potential avenue to create treatments that specifically deplete the ASC population.

6.3. Conclusions

ASC differentiation requires B cells to undergo extensive transcriptional and morphological changes. This transformation is tightly regulated by factors that drive the differentiation process and factors that maintain the B cell transcriptional network and is also influenced by the rate of B cell proliferation. In this thesis, I have shown that within the ASC gene signature, there are 4 genes (*Irf4*, *Prdm1*, *Hspa5* and *Sec61a1*) that are essential for *in vitro* generation of ASCs in response to LPS stimulation. Furthermore, I have identified 8 novel genes (*AB124611*, *Arhgef18*, *A43007G23Rik*, *Fam43a*, *Pold1*, *Ripk3*, *Rnf130* and *Rps6ka5*) that negatively regulate the rate of differentiation. I have identified 7 genes (*Cdv3*, *Hspa5*, *Irf4*, *Sec61a1*, *Selk*, *Sumo2* and *Vcp*) that positively regulate the rate of B cell division, 6 of which have not been previously demonstrated to influence proliferation of B cells including *Cdv3*, which has no previous link to proliferation in any cell type. This work has also revealed that there are 35 genes (*Bckdk*, *Bet1*, *Btd*, *Calr*, *Cacna1h*, *Ddost*, *Desi1*, *Dhdds*, *Dnajb11*, *Dnajc3*, *Dpagt1*, *Edem1*, *Ell2*, *Enpp1*, *Erlec1*, *Fcer1g*, *Fkbp2*, *Fkbp11*, *Fndc3a*, *Fos*, *Fut1*, *Irf4*, *Isg20*, *Prdm1*, *Qpctl*, *Sec61a1*, *Srprb*, *Tmem66*, *Tns3*, *Trabd*, *Tvp23b*, *Uba5*, *Xbp1*, *Yars* and *Zfyve21*) within the ASC gene signature that are essential for the efficient generation and secretion of antibodies, many of which have no previous association with the secretory process. Finally, I have uncovered a novel role for the transcription factor *Irf4* in the development of the peritoneal B-1a cell population. Together, this work highlights a number of regulators of the generation and function of populations within the B cell lineage that present potential novel avenues for the therapeutic manipulation of the humoral immune response

Bibliography

Ahearn, J.M., Fischer, M.B., Croix, D., Goerg, S., Ma, M., Xia, J., Zhou, X., Howard, R.G., Rothstein, T.L., and Carroll, M.C. (1996). Disruption of the Cr2 Locus Results in a Reduction in B-1a Cells and in an Impaired B Cell Response to T-Dependent Antigen. *Immunity* 4, 251-262.

Alugupalli, K.R., Leong, J.M., Woodland, R.T., Muramatsu, M., Honjo, T., and Gerstein, R.M. (2004). B1b Lymphocytes Confer T Cell-Independent Long-Lasting Immunity. *Immunity* 21, 379-390.

Amanna, I.J., Carlson, N.E., and Slifka, M.K. (2007). Duration of humoral immunity to common viral and vaccine antigens. *N Engl J Med* 357, 1903-1915.

Amanna, I.J., and Slifka, M.K. (2010). Mechanisms that determine plasma cell lifespan and the duration of humoral immunity. *Immunological reviews* 236, 125-138.

Ananieva, O., Darragh, J., Johansen, C., Carr, J.M., McIlrath, J., Park, J.M., Wingate, A., Monk, C.E., Toth, R., Santos, S.G., *et al.* (2008). The kinases MSK1 and MSK2 act as negative regulators of Toll-like receptor signaling. *Nature Immunology* 9, 1028-1036.

Anderson, M.K., Hernandez-Hoyos, G., Diamond, R.A., and Rothenberg, E.V. (1999). Precise developmental regulation of Ets family transcription factors during specification and commitment to the T cell lineage. *Development* 126, 3131-3148.

Arnold, L.W., Pennell, C.A., McCray, S.K., and Clarke, S.H. (1994). Development of B-1 cells: segregation of phosphatidyl choline-specific B cells to the B-1 population occurs after immunoglobulin gene expression. *The Journal of Experimental Medicine* 179, 1585-1595.

Bao, Y., Zhao, T.L., Zhang, Z.Q., Liang, X.L., Wang, Z.X., Xiong, Y., Lu, X., and Wang, L.H. (2020). High eukaryotic translation elongation factor 1 alpha 1 expression promotes proliferation and predicts poor prognosis in clear cell renal cell carcinoma. *Neoplasma* 67, 78-84.

Barberis, A., Widenhorn, K., Vitelli, L., and Busslinger, M. (1990). A novel B-cell lineage-specific transcription factor present at early but not late stages of differentiation. *Genes & Development* 4, 849-859.

Barton, K., Muthusamy, N., Fischer, C., Ting, C.-N., Walunas, T.L., Lanier, L.L., and Leiden, J.M. (1998). The Ets-1 Transcription Factor Is Required for the Development of Natural Killer Cells in Mice. *Immunity* 9, 555-563.

Barwick, B.G., Scharer, C.D., Martinez, R.J., Price, M.J., Wein, A.N., Haines, R.R., Bally, A.P.R., Kohlmeier, J.E., and Boss, J.M. (2018). B cell activation and plasma cell differentiation are inhibited by de novo DNA methylation. *Nature Communications* 9, 1900.

- Baumgarth, N., Herman, O.C., Jager, G.C., Brown, L., Herzenberg, L.A., and Herzenberg, L.A. (1999). Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proceedings of the National Academy of Sciences* 96, 2250-2255.
- Bergantini, L., d'Alessandro, M., Cameli, P., Vietri, L., Vagaggini, C., Perrone, A., Sestini, P., Frediani, B., and Bargagli, E. (2020). Effects of rituximab therapy on B cell differentiation and depletion. *Clinical Rheumatology* 39, 1415-1421.
- Berland, R., and Wortis, H.H. (2002). Origins and Functions of B-1 Cells with Notes on the Role of CD5. *Annual Review of Immunology* 20, 253-300.
- Bertolotti, A., Zhang, Y., Hendershot, L.M., Harding, H.P., and Ron, D. (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nature Cell Biology* 2, 326-332.
- Bhattacharya, D., Cheah, M.T., Franco, C.B., Hosen, N., Pin, C.L., Sha, W.C., and Weissman, I.L. (2007). Transcriptional Profiling of Antigen-Dependent Murine B Cell Differentiation and Memory Formation. *The Journal of Immunology* 179, 6808-6819.
- Bikah, G., Carey, J., Ciallella, J.R., Tarakhovsky, A., and Bondada, S. (1996). CD5-mediated negative regulation of antigen receptor-induced growth signals in B-1 B cells. *Science* 274, 1906-1909.
- Blink, E.J., Light, A., Kallies, A., Nutt, S.L., Hodgkin, P.D., and Tarlinton, D.M. (2005). Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. *The Journal of Experimental Medicine* 201, 545-554.
- Bollig, N., Brüstle, A., Kellner, K., Ackermann, W., Abass, E., Raifer, H., Camara, B., Brendel, C., Giel, G., Bothur, E., *et al.* (2012). Transcription factor IRF4 determines germinal center formation through follicular T-helper cell differentiation. *Proceedings of the National Academy of Sciences of the United States of America* 109, 8664-8669.
- Bories, J.-C., Willerford, D.M., Grévin, D., Davidson, L., Camus, A., Martin, P., Stéhelin, D., and Alt, F.W. (1995). Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene. *Nature* 377, 635-638.
- Braakman, I., and Hebert, D.N. (2013). Protein folding in the endoplasmic reticulum. *Cold Spring Harb Perspect Biol* 5, a013201.
- Brightbill, H., and Schlissel, M.S. (2009). The effects of c-Abl mutation on developing B cell differentiation and survival. *International immunology* 21, 575-585.
- Brinkmann, V., and Heusser, C.H. (1993). T Cell-Dependent Differentiation of Human B Cells into IgM, IgG, IgA, or IgE Plasma Cells: High Rate of Antibody Production by IgE Plasma Cells, but Limited Clonal Expansion of IgE Precursors. *Cellular Immunology* 152, 323-332.

Bros, M., Haas, K., Moll, L., and Grabbe, S. (2019). RhoA as a Key Regulator of Innate and Adaptive Immunity. *Cells* 8, 733.

Calfon, M., Zeng, H., Urano, F., Till, J.H., Hubbard, S.R., Harding, H.P., Clark, S.G., and Ron, D. (2002). IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* 415, 92-96.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of Histone H3 Lysine 27 Methylation in Polycomb-Group Silencing. *Science* 298, 1039-1043.

Carotta, S., Willis, S.N., Hasbold, J., Inouye, M., Pang, S.H., Emslie, D., Light, A., Chopin, M., Shi, W., Wang, H., *et al.* (2014). The transcription factors IRF8 and PU.1 negatively regulate plasma cell differentiation. *J Exp Med* 211, 2169-2181.

Cattoretti, G., Chang, C., Cechova, K., Zhang, J., Ye, B., Falini, B., Louie, D., Offit, K., Chaganti, R., and Dalla-Favera, R. (1995). BCL-6 protein is expressed in germinal-center B cells. *Blood* 86, 45-53.

Cattoretti, G., Shaknovich, R., Smith, P.M., Jäck, H.-M., Murty, V.V., and Alobeid, B. (2006). Stages of Germinal Center Transit Are Defined by B Cell Transcription Factor Coexpression and Relative Abundance. *The Journal of Immunology* 177, 6930-6939.

Cerutti, A., Cols, M., and Puga, I. (2013). Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nature Reviews Immunology* 13, 118-132.

Chan, V.W.F., Meng, F., Soriano, P., DeFranco, A.L., and Lowell, C.A. (1997). Characterization of the B Lymphocyte Populations in Lyn-Deficient Mice and the Role of Lyn in Signal Initiation and Down-Regulation. *Immunity* 7, 69-81.

Chan, W.F., Coughlan, H.D., Zhou, J.H.S., Keenan, C.R., Bediaga, N.G., Hodgkin, P.D., Smyth, G.K., Johanson, T.M., and Allan, R.S. (2019). Genome reconfiguration prior to mitosis shapes the generation of adaptive immunity. *bioRxiv*, 762757.

Chi, X., Li, Y., and Qiu, X. (2020). V(D)J recombination, somatic hypermutation and class switch recombination of immunoglobulins: mechanism and regulation. *Immunology* 160, 233-247.

Christianson, J.C., Shaler, T.A., Tyler, R.E., and Kopito, R.R. (2008). OS-9 and GRP94 deliver mutant α 1-antitrypsin to the Hrd1–SEL1L ubiquitin ligase complex for ERAD. *Nature cell biology* 10, 272-282.

Chu, V.T., Weber, T., Graf, R., Sommermann, T., Petsch, K., Sack, U., Volchkov, P., Rajewsky, K., and Kuhn, R. (2016). Efficient generation of Rosa26 knock-in mice using CRISPR/Cas9 in C57BL/6 zygotes. *BMC Biotechnol* 16, 4.

Chumley, M.J., Dal Porto, J.M., Kawaguchi, S., Cambier, J.C., Nemazee, D., and Hardy, R.R. (2000). A VH11Vk9 B Cell Antigen Receptor Drives Generation of CD5+ B Cells Both In Vivo and In Vitro. *The Journal of Immunology* 164, 4586-4593.

Cobaleda, C., Jochum, W., and Busslinger, M. (2007). Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors. *Nature* 449, 473-477.

Conde, C.D., Petronczki, Ö.Y., Baris, S., Willmann, K.L., Girardi, E., Salzer, E., Weitzer, S., Ardy, R.C., Krolo, A., Ijspeert, H., *et al.* (2019). Polymerase δ deficiency causes syndromic immunodeficiency with replicative stress. *The Journal of clinical investigation* 129, 4194-4206.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., *et al.* (2013). Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* 339, 819-823.

Cruciat, C.M., Hassler, C., and Niehrs, C. (2006). The MRH protein Erlectin is a member of the endoplasmic reticulum synexpression group and functions in N-glycan recognition. *J Biol Chem* 281, 12986-12993.

Cui, Y., Keles, S., Charbonnier, L.-M., Julé, A.M., Henderson, L., Celik, S.C., Reisli, I., Shen, C., Xie, W.J., Schmitz-Abe, K., *et al.* (2020). Combined immunodeficiency caused by a loss-of-function mutation in DNA polymerase delta 1. *Journal of Allergy and Clinical Immunology* 145, 391-401.e398.

Dapas, B., Pozzato, G., Zorzet, S., Capolla, S., Macor, P., Scaggiante, B., Coan, M., Guerra, C., Gnan, C., Gattei, V., *et al.* (2020). Effects of eEF1A1 targeting by aptamer/siRNA in chronic lymphocytic leukaemia cells. *International Journal of Pharmaceutics* 574, 118895.

Davis, C.W., Jackson, K.J.L., McCausland, M.M., Darce, J., Chang, C., Linderman, S.L., Chennareddy, C., Gerkin, R., Brown, S.J., Wrammert, J., *et al.* (2020). Influenza vaccine-induced human bone marrow plasma cells decline within a year after vaccination. *Science* 370, 237-241.

De Silva, N.S., Simonetti, G., Heise, N., and Klein, U. (2012). The diverse roles of IRF4 in late germinal center B-cell differentiation. *Immunological Reviews* 247, 73-92.

Deenick, E.K., Hasbold, J., and Hodgkin, P.D. (1999). Switching to IgG3, IgG2b, and IgA Is Division Linked and Independent, Revealing a Stochastic Framework for Describing Differentiation. *The Journal of Immunology* 163, 4707-4714.

Delogu, A., Schebesta, A., Sun, Q., Aschenbrenner, K., Perlot, T., and Busslinger, M. (2006). Gene Repression by Pax5 in B Cells Is Essential for Blood Cell Homeostasis and Is Reversed in Plasma Cells. *Immunity* 24, 269-281.

- Deltcheva, E., Chylinski, K., Sharma, C.M., Gonzales, K., Chao, Y., Pirzada, Z.A., Eckert, M.R., Vogel, J., and Charpentier, E. (2011). CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 471, 602.
- Dent, A.L., Shaffer, A.L., Yu, X., Allman, D., and Staudt, L.M. (1997). Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science* 276, 589-592.
- Doench, J.G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E.W., Donovan, K.F., Smith, I., Tothova, Z., Wilen, C., Orchard, R., *et al.* (2016). Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nature Biotechnology* 34, 184-191.
- Du, S., Zhou, J., Jia, Y., and Huang, K. (2010). SelK is a novel ER stress-regulated protein and protects HepG2 cells from ER stress agent-induced apoptosis. *Archives of Biochemistry and Biophysics* 502, 137-143.
- Eckert, V., Blank, M., Mazhari-Tabrizi, R., Mumberg, D., Funk, M., and Schwarz, R.T. (1998). Cloning and functional expression of the human GlcNAc-1-P transferase, the enzyme for the committed step of the dolichol cycle, by heterologous complementation in *Saccharomyces cerevisiae*. *Glycobiology* 8, 77-85.
- Eifler, K., and Vertegaal, A.C.O. (2015). SUMOylation-Mediated Regulation of Cell Cycle Progression and Cancer. *Trends in biochemical sciences* 40, 779-793.
- Endo, S., Zhang, Y.-W., Takahashi, S., and Koyama, T. (2003). Identification of human dehydrodolichyl diphosphate synthase gene. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* 1625, 291-295.
- Engel, P., Zhou, L.-J., Ord, D.C., Sato, S., Koller, B., and Tedder, T.F. (1995). Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity* 3, 39-50.
- Erdmann, F., Schäuble, N., Lang, S., Jung, M., Honigsmann, A., Ahmad, M., Dudek, J., Benedix, J., Harsman, A., Kopp, A., *et al.* (2011). Interaction of calmodulin with Sec61 α limits Ca(2+) leakage from the endoplasmic reticulum. *The EMBO Journal* 30, 17-31.
- Eyquem, S., Chemin, K., Fasseu, M., Chopin, M., Sigaux, F., Cumano, A., and Bories, J.-C. (2004). The development of early and mature B cells is impaired in mice deficient for the Ets-1 transcription factor. *European Journal of Immunology* 34, 3187-3196.
- Fairfax, K.A., Corcoran, L.M., Pridans, C., Huntington, N.D., Kallies, A., Nutt, S.L., and Tarlinton, D.M. (2007). Different kinetics of blimp-1 induction in B cell subsets revealed by reporter gene. *J Immunol* 178, 4104-4111.

- Feng, J., Wang, H., Shin, D.-M., Masiuk, M., Qi, C.-F., and Morse, H.C., 3rd (2011). IFN regulatory factor 8 restricts the size of the marginal zone and follicular B cell pools. *Journal of immunology (Baltimore, Md : 1950)* **186**, 1458-1466.
- Fukuda, T., Yoshida, T., Okada, S., Hatano, M., Miki, T., Ishibashi, K., Okabe, S., Koseki, H., Hirose, S., Taniguchi, M., *et al.* (1997). Disruption of the Bcl6 Gene Results in an Impaired Germinal Center Formation. *Journal of Experimental Medicine* **186**, 439-448.
- Fuxa, M., and Busslinger, M. (2007). Reporter Gene Insertions Reveal a Strictly B Lymphoid-Specific Expression Pattern of Pax5 in Support of Its B Cell Identity Function. *The Journal of Immunology* **178**, 3031-3037.
- Gallagher, D.N., and Haber, J.E. (2018). Repair of a site-specific DNA cleavage: old-school lessons for Cas9-mediated gene editing. *ACS chemical biology* **13**, 397-405.
- Garneau, J.E., Dupuis, M.-È., Villion, M., Romero, D.A., Barrangou, R., Boyaval, P., Fremaux, C., Horvath, P., Magadán, A.H., and Moineau, S. (2010). The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**, 67.
- Garrett-Sinha, L.A. (2013). Review of Ets1 structure, function, and roles in immunity. *Cellular and Molecular Life Sciences* **70**, 3375-3390.
- Gass, J.N., Gifford, N.M., and Brewer, J.W. (2002). Activation of an Unfolded Protein Response during Differentiation of Antibody-secreting B Cells. *Journal of Biological Chemistry* **277**, 49047-49054.
- Gass, J.N., Jiang, H.Y., Wek, R.C., and Brewer, J.W. (2008). The unfolded protein response of B-lymphocytes: PERK-independent development of antibody-secreting cells. *Mol Immunol* **45**, 1035-1043.
- Ghafoor, A., and Joseph, S.M. (2020). Making a Diagnosis of Common Variable Immunodeficiency: A Review. *Cureus* **12**, e6711-e6711.
- Ghosh, R., Wang, L., Wang, E.S., Perera, B.G.K., Igbaria, A., Morita, S., Prado, K., Thamsen, M., Caswell, D., Macias, H., *et al.* (2014). Allosteric inhibition of the IRE1 α RNase preserves cell viability and function during endoplasmic reticulum stress. *Cell* **158**, 534-548.
- Gilbert, L.A., Horlbeck, M.A., Adamson, B., Villalta, J.E., Chen, Y., Whitehead, E.H., Guimaraes, C., Panning, B., Ploegh, H.L., Bassik, M.C., *et al.* (2014). Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell* **159**, 647-661.
- Gloury, R., Zotos, D., Zuidschewoude, M., Masson, F., Liao, Y., Hasbold, J., Corcoran, L.M., Hodgkin, P.D., Belz, G.T., Shi, W., *et al.* (2016). Dynamic changes in Id3 and E-protein activity orchestrate germinal center and plasma cell development. *Journal of Experimental Medicine* **213**, 1095-1111.

Görlich, D., Prehn, S., Hartmann, E., Kalies, K.-U., and Rapoport, T.A. (1992). A mammalian homolog of SEC61p and SECYp is associated with ribosomes and nascent polypeptides during translocation. *Cell* 71, 489-503.

Graf, R., Seagal, J., Otipoby, K.L., Lam, K.P., Ayoub, S., Zhang, B., Sander, S., Chu, V.T., and Rajewsky, K. (2019). BCR-dependent lineage plasticity in mature B cells. *Science* 363, 748-753.

Griffin, D.O., Holodick, N.E., and Rothstein, T.L. (2011). Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70. *The Journal of experimental medicine* 208, 67-80.

Groisman, B., Shenkman, M., Ron, E., and Lederkremer, G.Z. (2011). Mannose trimming is required for delivery of a glycoprotein from EDEM1 to XTP3-B and to late endoplasmic reticulum-associated degradation steps. *The Journal of biological chemistry* 286, 1292-1300.

Grootjans, J., Kaser, A., Kaufman, R.J., and Blumberg, R.S. (2016). The unfolded protein response in immunity and inflammation. *Nature reviews Immunology* 16, 469-484.

Grötsch, B., Brachs, S., Lang, C., Luther, J., Derer, A., Schlötzer-Schrehardt, U., Bozec, A., Fillatreau, S., Berberich, I., Hobeika, E., *et al.* (2014). The AP-1 transcription factor Fra1 inhibits follicular B cell differentiation into plasma cells. *Journal of Experimental Medicine* 211, 2199-2212.

Guo, M., Price, M.J., Patterson, D.G., Barwick, B.G., Haines, R.R., Kania, A.K., Bradley, J.E., Randall, T.D., Boss, J.M., and Scharer, C.D. (2018). EZH2 Represses the B Cell Transcriptional Program and Regulates Antibody-Secreting Cell Metabolism and Antibody Production. *The Journal of Immunology* 200, 1039-1052.

Ha, Y.J., Jeong, J.H., Park, Y., and Lee, J.R. (2012). Increased p190RhoGEF expression in activated B cells correlates with the induction of the plasma cell differentiation. *Exp Mol Med* 44, 138-148.

Haas, K.M., Poe, J.C., Steeber, D.A., and Tedder, T.F. (2005). B-1a and B-1b Cells Exhibit Distinct Developmental Requirements and Have Unique Functional Roles in Innate and Adaptive Immunity to *S. pneumoniae*. *Immunity* 23, 7-18.

Hänzelmann, P., Galgenmüller, C., and Schindelin, H. (2019). Structure and Function of the AAA+ ATPase p97, a Key Player in Protein Homeostasis. *Subcell Biochem* 93, 221-272.

Hardin, J.D., Boast, S., Schwartzberg, P.L., Lee, G., Alt, F.W., Stall, A.M., and Goff, S.P. (1996). Abnormal Peripheral Lymphocyte Function in c-abl Mutant Mice. *Cellular Immunology* 172, 100-107.

Harding, H.P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000). Regulated Translation Initiation Controls Stress-Induced Gene Expression in Mammalian Cells. *Molecular Cell* 6, 1099-1108.

Harding, H.P., Zhang, Y., and Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* 397, 271-274.

Hasbold, J., Corcoran, L.M., Tarlinton, D.M., Tangye, S.G., and Hodgkin, P.D. (2004). Evidence from the generation of immunoglobulin G-secreting cells that stochastic mechanisms regulate lymphocyte differentiation. *Nat Immunol* 5, 55-63.

Hatzi, K., and Melnick, A. (2014). Breaking bad in the germinal center: how deregulation of BCL6 contributes to lymphomagenesis. *Trends in Molecular Medicine* 20, 343-352.

Hay, J.C., Hirling, H., and Scheller, R.H. (1996). Mammalian vesicle trafficking proteins of the endoplasmic reticulum and Golgi apparatus. *J Biol Chem* 271, 5671-5679.

Hayakawa, K., Carmack, C.E., Hyman, R., and Hardy, R.R. (1990). Natural autoantibodies to thymocytes: origin, VH genes, fine specificities, and the role of Thy-1 glycoprotein. *Journal of Experimental Medicine* 172, 869-878.

Hayakawa, K., Hardy, R.R., Herzenberg, L.A., and Herzenberg, L.A. (1985). Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. *Journal of Experimental Medicine* 161, 1554-1568.

Hayakawa, K., Hardy, R.R., Honda, M., Herzenberg, L.A., Steinberg, A.D., and Herzenberg, L.A. (1984). Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. *Proceedings of the National Academy of Sciences of the United States of America* 81, 2494-2498.

Hayakawa, K., Hardy, R.R., Parks, D.R., and Herzenberg, L.A. (1983). The "Ly-1 B" cell subpopulation in normal immunodeficient, and autoimmune mice. *The Journal of experimental medicine* 157, 202-218.

Hayakawa, K., Hardy, R.R., Stall, A.M., Herzenberg, L.A., and Herzenberg, L.A. (1986). Immunoglobulin-bearing B cells reconstitute and maintain the murine Ly-1 B cell lineage. *European Journal of Immunology* 16, 1313-1316.

Hayakawa, K., Li, Y.-S., Shinton, S.A., Bandi, S.R., Formica, A.M., Brill-Dashoff, J., and Hardy, R.R. (2019). Crucial Role of Increased Arid3a at the Pre-B and Immature B Cell Stages for B1a Cell Generation. *Frontiers in immunology* 10, 457-457.

Haze, K., Yoshida, H., Yanagi, H., Yura, T., and Mori, K. (1999). Mammalian Transcription Factor ATF6 Is Synthesized as a Transmembrane Protein and Activated by Proteolysis in Response to Endoplasmic Reticulum Stress. *Molecular Biology of the Cell* 10, 3787-3799.

Heise, N., De Silva, N.S., Silva, K., Carette, A., Simonetti, G., Pasparakis, M., and Klein, U. (2014). Germinal center B cell maintenance and differentiation are controlled by distinct NF- κ B transcription factor subunits. *Journal of Experimental Medicine* 211, 2103-2118.

Hetz, C., and Papa, F.R. (2018). The Unfolded Protein Response and Cell Fate Control. *Molecular Cell* 69, 169-181.

Hodgkin, P.D., Lee, J.H., and Lyons, A.B. (1996). B cell differentiation and isotype switching is related to division cycle number. *Journal of Experimental Medicine* 184, 277-281.

Horcher, M., Souabni, A., and Busslinger, M. (2001). Pax5/BSAP Maintains the Identity of B Cells in Late B Lymphopoiesis. *Immunity* 14, 779-790.

Hosokawa, N., Kamiya, Y., Kamiya, D., Kato, K., and Nagata, K. (2009). Human OS-9, a lectin required for glycoprotein endoplasmic reticulum-associated degradation, recognizes mannose-trimmed N-glycans. *Journal of Biological Chemistry* 284, 17061-17068.

Hosokawa, N., Wada, I., Hasegawa, K., Yorihuzi, T., Tremblay, L.O., Herscovics, A., and Nagata, K. (2001). A novel ER α -mannosidase-like protein accelerates ER-associated degradation. *EMBO reports* 2, 415-422.

Hu, C.C., Dougan, S.K., McGehee, A.M., Love, J.C., and Ploegh, H.L. (2009). XBP-1 regulates signal transduction, transcription factors and bone marrow colonization in B cells. *Embo j* 28, 1624-1636.

Huang, C., Geng, H., Boss, I., Wang, L., and Melnick, A. (2014). Cooperative transcriptional repression by BCL6 and BACH2 in germinal center B-cell differentiation. *Blood* 123, 1012-1020.

Huang, C., and Melnick, A. (2015). Mechanisms of action of BCL6 during germinal center B cell development. *Science China Life Sciences* 58, 1226-1232.

Humbert, P.O., and Corcoran, L.M. (1997). oct-2 gene disruption eliminates the peritoneal B-1 lymphocyte lineage and attenuates B-2 cell maturation and function. *The Journal of Immunology* 159, 5273-5284.

Iwakoshi, N.N., Lee, A.-H., Vallabhajosyula, P., Otipoby, K.L., Rajewsky, K., and Glimcher, L.H. (2003). Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nature Immunology* 4, 321.

Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D.H., and Sommer, T. (2002). Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. *Nat Cell Biol* 4, 134-139.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. (2012). A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* 337, 816-821.

Johanson, T.M., Lun, A.T.L., Coughlan, H.D., Tan, T., Smyth, G.K., Nutt, S.L., and Allan, R.S. (2018). Transcription-factor-mediated supervision of global genome architecture maintains B cell identity. *Nature Immunology* 19, 1257-1264.

- John, S., Russell, L., Chin, S.S., Luo, W., Oshima, R., and Garrett-Sinha, L.A. (2014). Transcription Factor Ets1, but Not the Closely Related Factor Ets2, Inhibits Antibody-Secreting Cell Differentiation. *Molecular and Cellular Biology* 34, 522-532.
- John, S.A., Clements, J.L., Russell, L.M., and Garrett-Sinha, L.A. (2008). Ets-1 Regulates Plasma Cell Differentiation by Interfering with the Activity of the Transcription Factor Blimp-1. *Journal of Biological Chemistry* 283, 951-962.
- Johnston, R.J., Poholek, A.C., DiToro, D., Yusuf, I., Eto, D., Barnett, B., Dent, A.L., Craft, J., and Crotty, S. (2009). Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science (New York, NY)* 325, 1006-1010.
- Kageyama, Y., and Katayama, N. (2020). Ontogeny of human B1 cells. *International Journal of Hematology* 111, 628-633.
- Kallies, A., Hasbold, J., Fairfax, K., Pridans, C., Emslie, D., McKenzie, B.S., Lew, A.M., Corcoran, L.M., Hodgkin, P.D., Tarlinton, D.M., *et al.* (2007). Initiation of plasma-cell differentiation is independent of the transcription factor Blimp-1. *Immunity* 26, 555-566.
- Kallies, A., Hasbold, J., Tarlinton, D.M., Dietrich, W., Corcoran, L.M., Hodgkin, P.D., and Nutt, S.L. (2004). Plasma Cell Ontogeny Defined by Quantitative Changes in Blimp-1 Expression. *The Journal of Experimental Medicine* 200, 967-977.
- Kallies, A., and Nutt, S.L. (2007). Terminal differentiation of lymphocytes depends on Blimp-1. *Curr Opin Immunol* 19, 156-162.
- Kantor, A.B., Stall, A.M., Adams, S., Herzenberg, L.A., and Herzenberg, L.A. (1992). Differential development of progenitor activity for three B-cell lineages. *Proceedings of the National Academy of Sciences* 89, 3320-3324.
- Karagiannis, P., Gilbert, A.E., Josephs, D.H., Ali, N., Dodev, T., Saul, L., Correa, I., Roberts, L., Beddowes, E., Koers, A., *et al.* (2013). IgG4 subclass antibodies impair antitumor immunity in melanoma. *The Journal of Clinical Investigation* 123, 1457-1474.
- Karagöz, G.E., Acosta-Alvear, D., Nguyen, H.T., Lee, C.P., Chu, F., and Walter, P. (2017). An unfolded protein-induced conformational switch activates mammalian IRE1. *Elife* 6.
- Karagöz, G.E., Acosta-Alvear, D., and Walter, P. (2019). The unfolded protein response: detecting and responding to fluctuations in the protein-folding capacity of the endoplasmic reticulum. *Cold Spring Harbor perspectives in biology* 11, a033886.
- Khan, W.N., Alt, F.W., Gerstein, R.M., Malynn, B.A., Larsson, I., Rathbun, G., Davidson, L., Müller, S., Kantor, A.B., Herzenberg, L.A., *et al.* (1995). Defective B cell development and function in Btk-deficient mice. *Immunity* 3, 283-299.

- Kienzler, A.K., Hargreaves, C.E., and Patel, S.Y. (2017). The role of genomics in common variable immunodeficiency disorders. *Clinical and experimental immunology* 188, 326-332.
- Kim, D., Paggi, J.M., Park, C., Bennett, C., and Salzberg, S.L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology* 37, 907-915.
- Kitabatake, M., Soma, M., Zhang, T., Kuwahara, K., Fukushima, Y., Nojima, T., Kitamura, D., and Sakaguchi, N. (2015). JNK Regulatory Molecule G5PR Induces IgG Autoantibody–Producing Plasmablasts from Peritoneal B1a Cells. *The Journal of Immunology*, 1401127.
- Klein, U., Casola, S., Cattoretti, G., Shen, Q., Lia, M., Mo, T., Ludwig, T., Rajewsky, K., and Dalla-Favera, R. (2006). Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. *Nat Immunol* 7, 773-782.
- Koike-Yusa, H., Li, Y., Tan, E.P., Velasco-Herrera, M.D.C., and Yusa, K. (2014). Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat Biotech* 32, 267-273.
- Kometani, K., Nakagawa, R., Shinnakasu, R., Kaji, T., Rybouchkin, A., Moriyama, S., Furukawa, K., Koseki, H., Takemori, T., and Kurosaki, T. (2013). Repression of the Transcription Factor Bach2 Contributes to Predisposition of IgG1 Memory B Cells toward Plasma Cell Differentiation. *Immunity* 39, 136-147.
- Konermann, S., Brigham, M.D., Trevino, A.E., Joung, J., Abudayyeh, O.O., Barcena, C., Hsu, P.D., Habib, N., Gootenberg, J.S., Nishimasu, H., *et al.* (2015). Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* 517, 583-588.
- Kong, S.E., Banks, C.A., Shilatifard, A., Conaway, J.W., and Conaway, R.C. (2005). ELL-associated factors 1 and 2 are positive regulators of RNA polymerase II elongation factor ELL. *Proceedings of the National Academy of Sciences* 102, 10094-10098.
- Korennykh, A.V., Egea, P.F., Korostelev, A.A., Finer-Moore, J., Zhang, C., Shokat, K.M., Stroud, R.M., and Walter, P. (2009). The unfolded protein response signals through high-order assembly of Ire1. *Nature* 457, 687-693.
- Kozlov, G., and Gehring, K. (2020). Calnexin cycle - structural features of the ER chaperone system. *Febs j.*
- Kozutsumi, Y., Segal, M., Normington, K., Gething, M.-J., and Sambrook, J. (1988). The presence of malformed proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature* 332, 462-464.
- Kreslavsky, T., Vilagos, B., Tagoh, H., Poliakova, D.K., Schwickert, T.A., Wohner, M., Jaritz, M., Weiss, S., Taneja, R., Rossner, M.J., *et al.* (2017). Essential role for the transcription factor Bhlhe41 in regulating the development, self-renewal and BCR repertoire of B-1a cells. *Nat Immunol* 18, 442-455.

- Kreslavsky, T., Wong, J.B., Fischer, M., Skok, J.A., and Busslinger, M. (2018). Control of B-1a cell development by instructive BCR signaling. *Curr Opin Immunol* 51, 24-31.
- Kurosaki, T., Kometani, K., and Ise, W. (2015). Memory B cells. *Nature Reviews Immunology* 15, 149-159.
- Kwon, H., Thierry-Mieg, D., Thierry-Mieg, J., Kim, H.P., Oh, J., Tunyaplin, C., Carotta, S., Donovan, C.E., Goldman, M.L., Taylor, P., *et al.* (2009). Analysis of interleukin-21-induced Prdm1 gene regulation reveals functional cooperation of STAT3 and IRF4 transcription factors. *Immunity* 31, 941-952.
- Kwon, K., Hutter, C., Sun, Q., Bilic, I., Cobaleda, C., Malin, S., and Busslinger, M. (2008). Instructive Role of the Transcription Factor E2A in Early B Lymphopoiesis and Germinal Center B Cell Development. *Immunity* 28, 751-762.
- Lang, S., Erdmann, F., Jung, M., Wagner, R., Cavalie, A., and Zimmermann, R. (2011). Sec61 complexes form ubiquitous ER Ca²⁺ leak channels. *Channels* 5, 228-235.
- Law, C.W., Chen, Y., Shi, W., and Smyth, G.K. (2014). voom: precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biology* 15, R29.
- Lee, A.-H., Iwakoshi, N.N., and Glimcher, L.H. (2003). XBP-1 Regulates a Subset of Endoplasmic Reticulum Resident Chaperone Genes in the Unfolded Protein Response. *Molecular and Cellular Biology* 23, 7448-7459.
- Lee, J.H., Park, K.J., Jang, J.K., Jeon, Y.H., Ko, K.Y., Kwon, J.H., Lee, S.-R., and Kim, I.Y. (2015). Selenoprotein S-dependent Selenoprotein K Binding to p97(VCP) Protein Is Essential for Endoplasmic Reticulum-associated Degradation. *The Journal of Biological Chemistry* 290, 29941-29952.
- Li, S., Wang, J., Hu, G., Aman, S., Li, B., Li, Y., Xia, K., Yang, Y., Ahmad, B., Wang, M., *et al.* (2020). SUMOylation of MCL1 protein enhances its stability by regulating the ubiquitin-proteasome pathway. *Cellular Signalling* 73, 109686.
- Li, W., Köster, J., Xu, H., Chen, C.-H., Xiao, T., Liu, J.S., Brown, M., and Liu, X.S. (2015). Quality control, modeling, and visualization of CRISPR screens with MAGeCK-VISPR. *Genome Biology* 16, 281.
- Li, W., Xu, H., Xiao, T., Cong, L., Love, M.I., Zhang, F., Irizarry, R.A., Liu, J.S., Brown, M., and Liu, X.S. (2014). MAGeCK enables robust identification of essential genes from genome-scale CRISPR/Cas9 knockout screens. *Genome Biology* 15, 554.
- Li, Y.-F., Xu, S., Huang, Y., Ou, X., and Lam, K.-P. (2017). Tyrosine kinase c-Abl regulates the survival of plasma cells. *Scientific reports* 7, 40133-40133.

Li, Z., Dong, X., Wang, Z., Liu, W., Deng, N., Ding, Y., Tang, L., Hla, T., Zeng, R., Li, L., *et al.* (2005). Regulation of PTEN by Rho small GTPases. *Nature Cell Biology* 7, 399-404.

Liao, Y., Smyth, G.K., and Shi, W. (2013). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923-930.

Liao, Y., Smyth, G.K., and Shi, W. (2019). The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. *Nucleic acids research* 47, e47-e47.

Lin, B., Saito, M., Sakakibara, Y., Hayashi, Y., Yanagisawa, M., and Iwamori, M. (2001). Characterization of Three Members of Murine α 1,2-Fucosyltransferases: Change in the Expression of the Se Gene in the Intestine of Mice after Administration of Microbes. *Archives of Biochemistry and Biophysics* 388, 207-215.

Lin, K.-I., Angelin-Duclos, C., Kuo, T.C., and Calame, K. (2002). Blimp-1-Dependent Repression of Pax-5 Is Required for Differentiation of B Cells to Immunoglobulin M-Secreting Plasma Cells. *Molecular and Cellular Biology* 22, 4771-4780.

Lin, L., Gerth, A.J., and Peng, S.L. (2004). Active inhibition of plasma cell development in resting B cells by microphthalmia-associated transcription factor. *The Journal of experimental medicine* 200, 115-122.

Lin, Y., Wong, K.-k., and Calame, K. (1997). Repression of c-myc Transcription by Blimp-1, an Inducer of Terminal B Cell Differentiation. *Science* 276, 596-599.

Liu, G.J., Jaritz, M., Wöhner, M., Agerer, B., Bergthaler, A., Malin, S.G., and Busslinger, M. (2020). Repression of the B cell identity factor Pax5 is not required for plasma cell development. *Journal of Experimental Medicine* 217.

Low, M.S.Y., Brodie, E.J., Fedele, P.L., Liao, Y., Grigoriadis, G., Strasser, A., Kallies, A., Willis, S.N., Tellier, J., Shi, W., *et al.* (2019). IRF4 Activity Is Required in Established Plasma Cells to Regulate Gene Transcription and Mitochondrial Homeostasis. *Cell Reports* 29, 2634-2645.e2635.

Lu, R., Medina, K.L., Lancki, D.W., and Singh, H. (2003). IRF-4,8 orchestrate the pre-B-to-B transition in lymphocyte development. *Genes & development* 17, 1703-1708.

Luo, W., Mayeux, J., Gutierrez, T., Russell, L., Getahun, A., Müller, J., Tedder, T., Parnes, J., Rickert, R., Nitschke, L., *et al.* (2014). A balance between B cell receptor and inhibitory receptor signaling controls plasma cell differentiation by maintaining optimal Ets1 levels. *Journal of immunology (Baltimore, Md : 1950)* 193, 909-920.

- Ma, K., Vattem, K.M., and Wek, R.C. (2002). Dimerization and Release of Molecular Chaperone Inhibition Facilitate Activation of Eukaryotic Initiation Factor-2 Kinase in Response to Endoplasmic Reticulum Stress. *Journal of Biological Chemistry* 277, 18728-18735.
- Ma, Y., Shimizu, Y., Mann, M.J., Jin, Y., and Hendershot, L.M. (2010). Plasma cell differentiation initiates a limited ER stress response by specifically suppressing the PERK-dependent branch of the unfolded protein response. *Cell Stress Chaperones* 15, 281-293.
- MacLennan, I.C.M., Toellner, K.-M., Cunningham, A.F., Serre, K., Sze, D.M.Y., Zúñiga, E., Cook, M.C., and Vinuesa, C.G. (2003). Extrafollicular antibody responses. *Immunological Reviews* 194, 8-18.
- Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-Guided Human Genome Engineering via Cas9. *Science* 339, 823-826.
- Mandelbaum, J., Bhagat, G., Tang, H., Mo, T., Brahmachary, M., Shen, Q., Chadburn, A., Rajewsky, K., Tarakhovsky, A., Pasqualucci, L., *et al.* (2010). BLIMP1 Is a Tumor Suppressor Gene Frequently Disrupted in Activated B Cell-like Diffuse Large B Cell Lymphoma. *Cancer Cell* 18, 568-579.
- Manz, R.A., Löhning, M., Cassese, G., Thiel, A., and Radbruch, A. (1998). Survival of long-lived plasma cells is independent of antigen. *International Immunology* 10, 1703-1711.
- Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. 2011 17, 3.
- Martincic, K., Alkan, S.A., Cheatle, A., Borghesi, L., and Milcarek, C. (2009). Transcription elongation factor ELL2 directs immunoglobulin secretion in plasma cells by stimulating altered RNA processing. *Nat Immunol* 10, 1102-1109.
- Medvedovic, J., Ebert, A., Tagoh, H., and Busslinger, M. (2011). Chapter 5 - Pax5: A Master Regulator of B Cell Development and Leukemogenesis. In *Advances in Immunology*, F.W. Alt, ed. (Academic Press), pp. 179-206.
- Mikkola, I., Heavey, B., Horcher, M., and Busslinger, M. (2002). Reversion of B Cell Commitment upon Loss of Pax5 Expression. *Science* 297, 110-113.
- Miller, J.D., Tajima, S., Lauffer, L., and Walter, P. (1995). The beta subunit of the signal recognition particle receptor is a transmembrane GTPase that anchors the alpha subunit, a peripheral membrane GTPase, to the endoplasmic reticulum membrane. *J Cell Biol* 128, 273-282.
- Minnich, M., Tagoh, H., Bönelt, P., Axelsson, E., Fischer, M., Cebolla, B., Tarakhovsky, A., Nutt, S.L., Jaritz, M., and Busslinger, M. (2016). Multifunctional role of the transcription factor Blimp-1 in coordinating plasma cell differentiation. *Nature Immunology* 17, 331.

- Mittrücker, H.-W., Matsuyama, T., Grossman, A., Kündig, T.M., Potter, J., Shahinian, A., Wakeham, A., Patterson, B., Ohashi, P.S., and Mak, T.W. (1997). Requirement for the Transcription Factor LSIRF/IRF4 for Mature B and T Lymphocyte Function. *Science* 275, 540-543.
- Mojica, F.J.M., Díez-Villaseñor, C., García-Martínez, J., and Almendros, C. (2009). Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* 155, 733-740.
- Montecino-Rodriguez, E., Fice, M., Casero, D., Berent-Maoz, B., Barber, Chad L., and Dorshkind, K. (2016). Distinct Genetic Networks Orchestrate the Emergence of Specific Waves of Fetal and Adult B-1 and B-2 Development. *Immunity* 45, 527-539.
- Montecino-Rodriguez, E., Leathers, H., and Dorshkind, K. (2006). Identification of a B-1 B cell–specified progenitor. *Nature Immunology* 7, 293-301.
- Morelli, E., Leone, E., Cantafio, M.E.G., Di Martino, M.T., Amodio, N., Biamonte, L., Gullà, A., Foresta, U., Pitari, M.R., Botta, C., *et al.* (2015). Selective targeting of IRF4 by synthetic microRNA-125b-5p mimics induces anti-multiple myeloma activity in vitro and in vivo. *Leukemia* 29, 2173-2183.
- Muto, A., Hoshino, H., Madisen, L., Yanai, N., Obinata, M., Karasuyama, H., Hayashi, N., Nakauchi, H., Yamamoto, M., Groudine, M., *et al.* (1998). Identification of Bach2 as a B-cell-specific partner for small Maf proteins that negatively regulate the immunoglobulin heavy chain gene 3' enhancer. *The EMBO Journal* 17, 5734-5743.
- Muto, A., Ochiai, K., Kimura, Y., Itoh-Nakadai, A., Calame, K.L., Ikebe, D., Tashiro, S., and Igarashi, K. (2010). Bach2 represses plasma cell gene regulatory network in B cells to promote antibody class switch. *The EMBO Journal* 29, 4048-4061.
- Muto, A., Tashiro, S., Nakajima, O., Hoshino, H., Takahashi, S., Sakoda, E., Ikebe, D., Yamamoto, M., and Igarashi, K. (2004). The transcriptional programme of antibody class switching involves the repressor Bach2. *Nature* 429, 566-571.
- Nam, S., and Lim, J.-S. (2016). Essential role of interferon regulatory factor 4 (IRF4) in immune cell development. *Archives of Pharmacal Research* 39, 1548-1555.
- Nera, K.-P., Kohonen, P., Narvi, E., Peippo, A., Mustonen, L., Terho, P., Koskela, K., Buerstedde, J.-M., and Lassila, O. (2006). Loss of Pax5 Promotes Plasma Cell Differentiation. *Immunity* 24, 283-293.
- Newton, K., Sun, X., and Dixit, V.M. (2004). Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. *Mol Cell Biol* 24, 1464-1469.

Nicolas, E., Golemis, E.A., and Arora, S. (2016). POLD1: Central mediator of DNA replication and repair, and implication in cancer and other pathologies. *Gene* 590, 128-141.

Ninagawa, S., Okada, T., Sumitomo, Y., Kamiya, Y., Kato, K., Horimoto, S., Ishikawa, T., Takeda, S., Sakuma, T., Yamamoto, T., *et al.* (2014). EDEM2 initiates mammalian glycoprotein ERAD by catalyzing the first mannose trimming step. *The Journal of Cell Biology* 206, 347-356.

Novoa, I., Zeng, H., Harding, H.P., and Ron, D. (2001). Feedback Inhibition of the Unfolded Protein Response by GADD34-Mediated Dephosphorylation of eIF2 α . *Journal of Cell Biology* 153, 1011-1022.

Nurieva, R.I., Chung, Y., Martinez, G.J., Yang, X.O., Tanaka, S., Matskevitch, T.D., Wang, Y.-H., and Dong, C. (2009). Bcl6 mediates the development of T follicular helper cells. *Science (New York, NY)* 325, 1001-1005.

Nutt, S.L., Heavey, B., Rolink, A.G., and Busslinger, M. (1999). Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature* 401, 556-562.

Nutt, S.L., Hodgkin, P.D., Tarlinton, D.M., and Corcoran, L.M. (2015). The generation of antibody-secreting plasma cells. *Nat Rev Immunol* 15, 160-171.

Nutt, S.L., Morrison, A.M., Dörfler, P., Rolink, A., and Busslinger, M. (1998). Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. *The EMBO Journal* 17, 2319-2333.

Nutt, S.L., Taubenheim, N., Hasbold, J., Corcoran, L.M., and Hodgkin, P.D. (2011). The genetic network controlling plasma cell differentiation. *Semin Immunol* 23, 341-349.

Nutt, S.L., Urbánek, P., Rolink, A., and Busslinger, M. (1997). Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes & Development* 11, 476-491.

O'Keefe, T.L., Williams, G.T., Davies, S.L., and Neuberger, M.S. (1996). Hyperresponsive B Cells in CD22-Deficient Mice. *Science* 274, 798-801.

Ochiai, K., Katoh, Y., Ikura, T., Hoshikawa, Y., Noda, T., Karasuyama, H., Tashiro, S., Muto, A., and Igarashi, K. (2006). Plasmacytic Transcription Factor Blimp-1 Is Repressed by Bach2 in B Cells. *Journal of Biological Chemistry* 281, 38226-38234.

Ochiai, K., Maienschein-Cline, M., Simonetti, G., Chen, J., Rosenthal, R., Brink, R., Chong, Anita S., Klein, U., Dinner, Aaron R., Singh, H., *et al.* (2013). Transcriptional Regulation of Germinal Center B and Plasma Cell Fates by Dynamical Control of IRF4. *Immunity* 38, 918-929.

- Ochiai, K., Muto, A., Tanaka, H., Takahashi, S., and Igarashi, K. (2008). Regulation of the plasma cell transcription factor Blimp-1 gene by Bach2 and Bcl6. *International Immunology* 20, 453-460.
- Oda, Y., Hosokawa, N., Wada, I., and Nagata, K. (2003). EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. *Science* 299, 1394-1397.
- Ohkubo, Y., Arima, M., Arguni, E., Okada, S., Yamashita, K., Asari, S., Obata, S., Sakamoto, A., Hatano, M., O-Wang, J., *et al.* (2005). A Role for c-fos/Activator Protein 1 in B Lymphocyte Terminal Differentiation. *The Journal of Immunology* 174, 7703-7710.
- Oliver, A.M., Martin, F., Gartland, G.L., Carter, R.H., and Kearney, J.F. (1997). Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. *Eur J Immunol* 27, 2366-2374.
- Oliver, A.M., Martin, F., and Kearney, J.F. (1999). IgM^{high}CD21^{high} Lymphocytes Enriched in the Splenic Marginal Zone Generate Effector Cells More Rapidly Than the Bulk of Follicular B Cells. *The Journal of Immunology* 162, 7198-7207.
- Pan, C., Baumgarth, N., and Parnes, J.R. (1999). CD72-Deficient Mice Reveal Nonredundant Roles of CD72 in B Cell Development and Activation. *Immunity* 11, 495-506.
- Pancheri, E., Guglielmi, V., Wilczynski, G.M., Malatesta, M., Tonin, P., Tomelleri, G., Nowis, D., and Vattermi, G. (2020). Non-Hematologic Toxicity of Bortezomib in Multiple Myeloma: The Neuromuscular and Cardiovascular Adverse Effects. *Cancers (Basel)* 12.
- Pappu, R., Cheng, A.M., Li, B., Gong, Q., Chiu, C., Griffin, N., White, M., Sleckman, B.P., and Chan, A.C. (1999). Requirement for B Cell Linker Protein (BLNK) in B Cell Development. *Science* 286, 1949-1954.
- Park, K.S., Bayles, I., Szlachta-McGinn, A., Paul, J., Boiko, J., Santos, P., Liu, J., Wang, Z., Borghesi, L., and Milcarek, C. (2014). Transcription Elongation Factor ELL2 Drives Ig Secretory-Specific mRNA Production and the Unfolded Protein Response. *The Journal of Immunology* 193, 4663-4674.
- Pathak, S., Ma, S., Trinh, L., and Lu, R. (2008). A role for interferon regulatory factor 4 in receptor editing. *Mol Cell Biol* 28, 2815-2824.
- Pedersen, G.K., Àdori, M., Khoenkhoen, S., Dosenovic, P., Beutler, B., and Karlsson Hedestam, G.B. (2014). B-1a transitional cells are phenotypically distinct and are lacking in mice deficient in IkbNS. *Proceedings of the National Academy of Sciences* 111, E4119-E4126.
- Pennell, C.A., Mercolino, T.J., Grdina, T.A., Arnold, L.W., Haughton, G., and Clarke, S.H. (1989a). Biased immunoglobulin variable region gene expression by Ly-1 B cells due to clonal selection. *European Journal of Immunology* 19, 1289-1295.

- Pennell, C.A., Sheehan, K.M., Brodeur, P.H., and Clarke, S.H. (1989b). Organization and expression of *vh* gene families preferentially expressed by *ly-1+* (*cd5*) b cells. *European Journal of Immunology* 19, 2115-2121.
- Peperzak, V., Vikström, I., Walker, J., Glaser, S.P., LePage, M., Coquery, C.M., Erickson, L.D., Fairfax, K., Mackay, F., Strasser, A., *et al.* (2013). *Mcl-1* is essential for the survival of plasma cells. *Nature immunology* 14, 290-297.
- Peterson, M.L. (2011). Immunoglobulin heavy chain gene regulation through polyadenylation and splicing competition. *Wiley Interdiscip Rev RNA* 2, 92-105.
- Phan, T.G., Paus, D., Chan, T.D., Turner, M.L., Nutt, S.L., Basten, A., and Brink, R. (2006). High affinity germinal center B cells are actively selected into the plasma cell compartment. *Journal of Experimental Medicine* 203, 2419-2424.
- Pillai, S., and Cariappa, A. (2009). The follicular versus marginal zone B lymphocyte cell fate decision. *Nature Reviews Immunology* 9, 767-777.
- Pincus, D., Chevalier, M.W., Aragón, T., van Anken, E., Vidal, S.E., El-Samad, H., and Walter, P. (2010). BiP binding to the ER-stress sensor *Ire1* tunes the homeostatic behavior of the unfolded protein response. *PLoS Biol* 8, e1000415.
- Piskurich, J.F., Lin, K.I., Lin, Y., Wang, Y., Ting, J.P.Y., and Calame, K. (2000). *BLIMP-1* mediates extinction of major histocompatibility class II transactivator expression in plasma cells. *Nature Immunology* 1, 526-532.
- Polli, M., Dakic, A., Light, A., Wu, L., Tarlinton, D.M., and Nutt, S.L. (2005). The development of functional B lymphocytes in conditional *PU.1* knock-out mice. *Blood* 106, 2083-2090.
- Preston, G.M., and Brodsky, J.L. (2017). The evolving role of ubiquitin modification in endoplasmic reticulum-associated degradation. *Biochemical Journal* 474, 445-469.
- Pridans, C., Holmes, M.L., Polli, M., Wettenhall, J.M., Dakic, A., Corcoran, L.M., Smyth, G.K., and Nutt, S.L. (2008). Identification of *Pax5* target genes in early B cell differentiation. *J Immunol* 180, 1719-1728.
- Rabinovich, E., Kerem, A., Fröhlich, K.-U., Diamant, N., and Bar-Nun, S. (2002). AAA-ATPase *p97/Cdc48p*, a Cytosolic Chaperone Required for Endoplasmic Reticulum-Associated Protein Degradation. *Molecular and Cellular Biology* 22, 626-634.
- Rauschmeier, R., Gustafsson, C., Reinhardt, A., A-Gonzalez, N., Tortola, L., Cansever, D., Subramanian, S., Taneja, R., Rossner, M.J., Sieweke, M.H., *et al.* (2019). *Bhlhe40* and *Bhlhe41* transcription factors regulate alveolar macrophage self-renewal and identity. *The EMBO journal* 38, e101233-e101233.

Reimold, A.M., Iwakoshi, N.N., Manis, J., Vallabhajosyula, P., Szomolanyi-Tsuda, E., Gravallese, E.M., Friend, D., Grusby, M.J., Alt, F., and Glimcher, L.H. (2001). Plasma cell differentiation requires the transcription factor XBP-1. *Nature* 412, 300-307.

Ricker, E., Chowdhury, L., Yi, W., and Pernis, A.B. (2016). The RhoA-ROCK pathway in the regulation of T and B cell responses. *F1000Research* 5, F1000 Faculty Rev-2295.

Rickert, R.C., Rajewsky, K., and Roes, J. (1995). Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature* 376, 352-355.

Roboti, P., and High, S. (2012). The oligosaccharyltransferase subunits OST48, DAD1 and KCP2 function as ubiquitous and selective modulators of mammalian N-glycosylation. *J Cell Sci* 125, 3474-3484.

Rolink, A.G., Nutt, S.L., Melchers, F., and Busslinger, M. (1999). Long-term in vivo reconstitution of T-cell development by Pax5-deficient B-cell progenitors. *Nature* 401, 603-606.

Rothstein, T.L., Griffin, D.O., Holodick, N.E., Quach, T.D., and Kaku, H. (2013). Human B-1 cells take the stage. *Annals of the New York Academy of Sciences* 1285, 97-114.

Roy, K., Mitchell, S., Liu, Y., Ohta, S., Lin, Y.-s., Metzger, M.O., Nutt, S.L., and Hoffmann, A. (2019). A Regulatory Circuit Controlling the Dynamics of NFκB cRel Transitions B Cells from Proliferation to Plasma Cell Differentiation. *Immunity* 50, 616-628.e616.

Saelee, P., Kearly, A., Nutt, S.L., and Garrett-Sinha, L.A. (2017). Genome-Wide Identification of Target Genes for the Key B Cell Transcription Factor Ets1. *Frontiers in Immunology* 8.

Saito, M., Gao, J., Basso, K., Kitagawa, Y., Smith, P.M., Bhagat, G., Pernis, A., Pasqualucci, L., and Dalla-Favera, R. (2007). A Signaling Pathway Mediating Downregulation of BCL6 in Germinal Center B Cells Is Blocked by BCL6 Gene Alterations in B Cell Lymphoma. *Cancer Cell* 12, 280-292.

Sato, S., Miller, A.S., Inaoki, M., Bock, C.B., Jansen, P.J., Tang, M.L., and Tedder, T.F. (1996a). CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. *Immunity* 5, 551-562.

Sato, S., Ono, N., Steeber, D.A., Pisetsky, D.S., and Tedder, T.F. (1996b). CD19 regulates B lymphocyte signaling thresholds critical for the development of B-1 lineage cells and autoimmunity. *The Journal of Immunology* 157, 4371-4378.

Savage, H.P., and Baumgarth, N. (2015). Characteristics of natural antibody-secreting cells. *Annals of the New York Academy of Sciences* 1362, 132-142.

- Scharer, C.D., Barwick, B.G., Guo, M., Bally, A.P.R., and Boss, J.M. (2018). Plasma cell differentiation is controlled by multiple cell division-coupled epigenetic programs. *Nature Communications* 9, 1698.
- Scharer, C.D., Patterson, D.G., Mi, T., Price, M.J., Hicks, S.L., and Boss, J.M. (2020). Antibody-secreting cell destiny emerges during the initial stages of B-cell activation. *Nature communications* 11, 3989-3989.
- Schebesta, A., McManus, S., Salvagiotto, G., Delogu, A., Busslinger, G.A., and Busslinger, M. (2007). Transcription Factor Pax5 Activates the Chromatin of Key Genes Involved in B Cell Signaling, Adhesion, Migration, and Immune Function. *Immunity* 27, 49-63.
- Schebesta, M., Pfeffer, P.L., and Busslinger, M. (2002). Control of Pre-BCR Signaling by Pax5-Dependent Activation of the BLNK Gene. *Immunity* 17, 473-485.
- Schliephake, D.E., and Schimpl, A. (1996). Blimp-1 overcomes the block in IgM secretion in lipopolysaccharide / anti- μ F(ab')₂-co-stimulated B lymphocytes. *European Journal of Immunology* 26, 268-271.
- Schmidlin, H., Diehl, S.A., Nagasawa, M., Scheeren, F.A., Schotte, R., Uittenbogaart, C.H., Spits, H., and Blom, B. (2008). Spi-B inhibits human plasma cell differentiation by repressing BLIMP1 and XBP-1 expression. *Blood* 112, 1804-1812.
- Schubert, D., Klein, M.-C., Hassdenteufel, S., Caballero-Oteyza, A., Yang, L., Proietti, M., Bulashevskaya, A., Kemming, J., Kühn, J., Winzer, S., *et al.* (2018). Plasma cell deficiency in human subjects with heterozygous mutations in Sec61 translocon alpha 1 subunit (SEC61A1). *Journal of Allergy and Clinical Immunology* 141, 1427-1438.
- Sciammas, R., Shaffer, A.L., Schatz, J.H., Zhao, H., Staudt, L.M., and Singh, H. (2006). Graded Expression of Interferon Regulatory Factor-4 Coordinates Isotype Switching with Plasma Cell Differentiation. *Immunity* 25, 225-236.
- Setz, C.S., Hug, E., Khadour, A., Abdelrasoul, H., Bilal, M., Hobeika, E., and Jumaa, H. (2018). PI3K-Mediated Blimp-1 Activation Controls B Cell Selection and Homeostasis. *Cell Reports* 24, 391-405.
- Shaffer, A.L., Emre, N.C., Lamy, L., Ngo, V.N., Wright, G., Xiao, W., Powell, J., Dave, S., Yu, X., Zhao, H., *et al.* (2008). IRF4 addiction in multiple myeloma. *Nature* 454, 226-231.
- Shaffer, A.L., Lin, K.-I., Kuo, T.C., Yu, X., Hurt, E.M., Rosenwald, A., Giltane, J.M., Yang, L., Zhao, H., Calame, K., *et al.* (2002). Blimp-1 Orchestrates Plasma Cell Differentiation by Extinguishing the Mature B Cell Gene Expression Program. *Immunity* 17, 51-62.

Shaffer, A.L., Shapiro-Shelef, M., Iwakoshi, N.N., Lee, A.H., Qian, S.B., Zhao, H., Yu, X., Yang, L., Tan, B.K., Rosenwald, A., *et al.* (2004). XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity* 21, 81-93.

Shaffer, A.L., Yu, X., He, Y., Boldrick, J., Chan, E.P., and Staudt, L.M. (2000). BCL-6 Represses Genes that Function in Lymphocyte Differentiation, Inflammation, and Cell Cycle Control. *Immunity* 13, 199-212.

Shalapour, S., Font-Burgada, J., Di Caro, G., Zhong, Z., Sanchez-Lopez, E., Dhar, D., Willimsky, G., Ammirante, M., Strasner, A., Hansel, D.E., *et al.* (2015). Immunosuppressive plasma cells impede T-cell-dependent immunogenic chemotherapy. *Nature* 521, 94-98.

Shapiro-Shelef, M., Lin, K.-I., McHeyzer-Williams, L.J., Liao, J., McHeyzer-Williams, M.G., and Calame, K. (2003). Blimp-1 Is Required for the Formation of Immunoglobulin Secreting Plasma Cells and Pre-Plasma Memory B Cells. *Immunity* 19, 607-620.

Sharonov, G.V., Serebrovskaya, E.O., Yuzhakova, D.V., Britanova, O.V., and Chudakov, D.M. (2020). B cells, plasma cells and antibody repertoires in the tumour microenvironment. *Nature Reviews Immunology* 20, 294-307.

Shchedrina, V.A., Everley, R.A., Zhang, Y., Gygi, S.P., Hatfield, D.L., and Gladyshev, V.N. (2011). Selenoprotein K binds multiprotein complexes and is involved in the regulation of endoplasmic reticulum homeostasis. *The Journal of biological chemistry* 286, 42937-42948.

Shen, J., Chen, X., Hendershot, L., and Prywes, R. (2002). ER Stress Regulation of ATF6 Localization by Dissociation of BiP/GRP78 Binding and Unmasking of Golgi Localization Signals. *Developmental Cell* 3, 99-111.

Shen, Y., and Hendershot, L.M. (2005). ERdj3, a stress-inducible endoplasmic reticulum DnaJ homologue, serves as a cofactor for BiP's interactions with unfolded substrates. *Mol Biol Cell* 16, 40-50.

Shenkman, M., and Lederkremer, G.Z. (2019). Compartmentalization and Selective Tagging for Disposal of Misfolded Glycoproteins. *Trends in Biochemical Sciences* 44, 827-836.

Shenkman, M., Ron, E., Yehuda, R., Benyair, R., Khalaila, I., and Lederkremer, G.Z. (2018). Mannosidase activity of EDEM1 and EDEM2 depends on an unfolded state of their glycoprotein substrates. *Communications Biology* 1, 172.

Shi, W., Liao, Y., Willis, S.N., Taubenheim, N., Inouye, M., Tarlinton, D.M., Smyth, G.K., Hodgkin, P.D., Nutt, S.L., and Corcoran, L.M. (2015). Transcriptional profiling of mouse B cell terminal differentiation defines a signature for antibody-secreting plasma cells. *Nat Immunol* 16, 663-673.

Shinnakasu, R., Inoue, T., Kometani, K., Moriyama, S., Adachi, Y., Nakayama, M., Takahashi, Y., Fukuyama, H., Okada, T., and Kurosaki, T. (2016). Regulated selection of germinal-center cells into the memory B cell compartment. *Nature Immunology* 17, 861-869.

Simonetti, G., Carette, A., Silva, K., Wang, H., De Silva, N.S., Heise, N., Siebel, C.W., Shlomchik, M.J., and Klein, U. (2013). IRF4 controls the positioning of mature B cells in the lymphoid microenvironments by regulating NOTCH2 expression and activity. *Journal of Experimental Medicine* 210, 2887-2902.

Slifka, M.K., Antia, R., Whitmire, J.K., and Ahmed, R. (1998). Humoral Immunity Due to Long-Lived Plasma Cells. *Immunity* 8, 363-372.

Smith, F.L., and Baumgarth, N. (2019). B-1 cell responses to infections. *Current opinion in immunology* 57, 23-31.

Smith, M.H., Ploegh, H.L., and Weissman, J.S. (2011). Road to ruin: targeting proteins for degradation in the endoplasmic reticulum. *Science (New York, NY)* 334, 1086-1090.

Snapper, C.M., Yamada, H., Smoot, D., Sneed, R., Lees, A., and Mond, J.J. (1993). Comparative in vitro analysis of proliferation, Ig secretion, and Ig class switching by murine marginal zone and follicular B cells. *The Journal of Immunology* 150, 2737-2745.

Song, J., Hong, P., Liu, C., Zhang, Y., Wang, J., and Wang, P. (2015). Human POLD1 modulates cell cycle progression and DNA damage repair. *BMC Biochem* 16, 14-14.

Stall, A.M., Adams, S., Herzenberg, L.A., and Kantor, A.B. (1992). Characteristics and Development of the Murine B-1b (Ly-1 B Sister) Cell Population. *Annals of the New York Academy of Sciences* 651, 33-43.

Storti, P., Costa, F., Marchica, V., Burroughs-Garcia, J., Dalla Palma, B., Toscani, D., Eufemiese, R.A., and Giuliani, N. (2020). Novel Approaches to Improve Myeloma Cell Killing by Monoclonal Antibodies. *J Clin Med* 9.

Suan, D., Sundling, C., and Brink, R. (2017). Plasma cell and memory B cell differentiation from the germinal center. *Current Opinion in Immunology* 45, 97-102.

Sun, Z., and Brodsky, J.L. (2019). Protein quality control in the secretory pathway. *The Journal of cell biology* 218, 3171-3187.

Sunshine, A., Goich, D., Stith, A., Sortino, K., Dalton, J., Metcalfe, S., Svensson, E.C., and Garrett-Sinha, L.A. (2019). Ets1 Controls the Development of B Cell Autoimmune Responses in a Cell-Intrinsic Manner. *Immunohorizons* 3, 331-340.

Tangye, S.G., Avery, D.T., Deenick, E.K., and Hodgkin, P.D. (2003a). Intrinsic differences in the proliferation of naive and memory human B cells as a mechanism for enhanced secondary immune responses. *J Immunol* 170, 686-694.

Tangye, S.G., Avery, D.T., and Hodgkin, P.D. (2003b). A division-linked mechanism for the rapid generation of Ig-secreting cells from human memory B cells. *J Immunol* 170, 261-269.

Tarakhovsky, A., Turner, M., Schaal, S., Mee, P.J., Duddy, L.P., Rajewsky, K., and Tybulewicz, V.L.J. (1995). Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. *Nature* 374, 467-470.

Tarlinton, D.M., McLean, M., and Nossal, G.J.V. (1995). B1 and B2 cells differ in their potential to switch immunoglobulin isotype. *European Journal of Immunology* 25, 3388-3393.

Taubenheim, N., Tarlinton, D.M., Crawford, S., Corcoran, L.M., Hodgkin, P.D., and Nutt, S.L. (2012). High Rate of Antibody Secretion Is not Integral to Plasma Cell Differentiation as Revealed by XBP-1 Deficiency. *The Journal of Immunology* 189, 3328-3338.

Tellier, J., Shi, W., Minnich, M., Liao, Y., Crawford, S., Smyth, G.K., Kallies, A., Busslinger, M., and Nutt, S.L. (2016). Blimp-1 controls plasma cell function through the regulation of immunoglobulin secretion and the unfolded protein response. *Nat Immunol* 17, 323-330.

Thévenin, C., Nutt, S.L., and Busslinger, M. (1998). Early Function of Pax5 (BSAP) before the Pre-B Cell Receptor Stage of B Lymphopoiesis. *Journal of Experimental Medicine* 188, 735-744.

Thurlings, I., and de Bruin, A. (2016). E2F Transcription Factors Control the Roller Coaster Ride of Cell Cycle Gene Expression. In *Cell Cycle Oscillators: Methods and Protocols*, A.S. Coutts, and L. Weston, eds. (New York, NY: Springer New York), pp. 71-88.

Todd, D.J., McHeyzer-Williams, L.J., Kowal, C., Lee, A.-H., Volpe, B.T., Diamond, B., McHeyzer-Williams, M.G., and Glimcher, L.H. (2009). XBP1 governs late events in plasma cell differentiation and is not required for antigen-specific memory B cell development. *The Journal of Experimental Medicine* 206, 2151-2159.

Trezise, S., Karnowski, A., Fedele, P.L., Mithraprabhu, S., Liao, Y., D'Costa, K., Kueh, A.J., Hardy, M.P., Owczarek, C.M., Herold, M.J., *et al.* (2018). Mining the Plasma Cell Transcriptome for Novel Cell Surface Proteins. *Int J Mol Sci* 19.

Tsuchiya, K., Kawano, Y., Kojima, T., Nagata, K., Takao, T., Okada, M., Shinohara, H., Maki, K., Toyama-Sorimachi, N., Miyasaka, N., *et al.* (2003). Molecular cloning and characterization of TPP36 and its isoform TPP32, novel substrates of Abl tyrosine kinase. *FEBS Letters* 537, 203-209.

- Tumini, E., Barroso, S., Calero, C.P., and Aguilera, A. (2016). Roles of human POLD1 and POLD3 in genome stability. *Scientific reports* 6, 38873-38873.
- Tunyaplin, C., Shaffer, A.L., Angelin-Duclos, C.D., Yu, X., Staudt, L.M., and Calame, K.L. (2004). Direct Repression of *Prdm1* by Bcl-6 Inhibits Plasmacytic Differentiation. *The Journal of Immunology* 173, 1158-1165.
- Turner, C.A., Jr., Mack, D.H., and Davis, M.M. (1994). Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell* 77, 297-306.
- Urbánek, P., Wang, Z.-Q., Fetka, I., Wagner, E.F., and Busslinger, M. (1994). Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell* 79, 901-912.
- van Anken, E., Romijn, E.P., Maggioni, C., Mezghrani, A., Sitia, R., Braakman, I., and Heck, A.J.R. (2003). Sequential Waves of Functionally Related Proteins Are Expressed When B Cells Prepare for Antibody Secretion. *Immunity* 18, 243-253.
- Varki, A. (2017). Biological roles of glycans. *Glycobiology* 27, 3-49.
- Verma, S., Hoffmann, F.W., Kumar, M., Huang, Z., Roe, K., Nguyen-Wu, E., Hashimoto, A.S., and Hoffmann, P.R. (2011). Selenoprotein K knockout mice exhibit deficient calcium flux in immune cells and impaired immune responses. *Journal of immunology (Baltimore, Md : 1950)* 186, 2127-2137.
- Victoria, G.D., Schwickert, T.A., Fooksman, D.R., Kamphorst, A.O., Meyer-Hermann, M., Dustin, M.L., and Nussenzweig, M.C. (2010). Germinal Center Dynamics Revealed by Multiphoton Microscopy with a Photoactivatable Fluorescent Reporter. *Cell* 143, 592-605.
- Vikstrom, I., Carotta, S., Lüthje, K., Peperzak, V., Jost, P.J., Glaser, S., Busslinger, M., Bouillet, P., Strasser, A., Nutt, S.L., *et al.* (2010). Mcl-1 is essential for germinal center formation and B cell memory. *Science (New York, NY)* 330, 1095-1099.
- Vinuesa, C.G., Linterman, M.A., Yu, D., and MacLennan, I.C.M. (2016). Follicular Helper T Cells. *Annual Review of Immunology* 34, 335-368.
- Wang, B., Wang, M., Zhang, W., Xiao, T., Chen, C.H., Wu, A., Wu, F., Traugh, N., Wang, X., Li, Z., *et al.* (2019). Integrative analysis of pooled CRISPR genetic screens using MAGeCKFlute. *Nat Protoc* 14, 756-780.
- Wang, D., John, S.A., Clements, J.L., Percy, D.H., Barton, K.P., and Garrett-Sinha, L.A. (2005). Ets-1 deficiency leads to altered B cell differentiation, hyperresponsiveness to TLR9 and autoimmune disease. *International Immunology* 17, 1179-1191.
- Wang, J., Lee, J., Liem, D., and Ping, P. (2017). HSPA5 Gene encoding Hsp70 chaperone BiP in the endoplasmic reticulum. *Gene* 618, 14-23.

- Wang, L., Wansleeben, C., Zhao, S., Miao, P., Paschen, W., and Yang, W. (2014). SUMO2 is essential while SUMO3 is dispensable for mouse embryonic development. *EMBO Reports* 15, 878-885.
- Wang, P., Li, J., Tao, J., and Sha, B. (2018). The luminal domain of the ER stress sensor protein PERK binds misfolded proteins and thereby triggers PERK oligomerization. *The Journal of biological chemistry* 293, 4110-4121.
- Welinder, C., Jirström, K., Lehn, S., Nodin, B., Marko-Varga, G., Blixt, O., Danielsson, L., and Jansson, B. (2016). Intra-tumour IgA1 is common in cancer and is correlated with poor prognosis in bladder cancer. *Heliyon* 2, e00143.
- Wiedenheft, B., Sternberg, S.H., and Doudna, J.A. (2012). RNA-guided genetic silencing systems in bacteria and archaea. *Nature* 482, 331.
- Willis, S.N., Good-Jacobson, K.L., Curtis, J., Light, A., Tellier, J., Shi, W., Smyth, G.K., Tarlinton, D.M., Belz, G.T., Corcoran, L.M., *et al.* (2014). Transcription Factor IRF4 Regulates Germinal Center Cell Formation through a B Cell–Intrinsic Mechanism. *The Journal of Immunology* 192, 3200-3206.
- Willis, S.N., Tellier, J., Liao, Y., Trezise, S., Light, A., O'Donnell, K., Garrett-Sinha, L.A., Shi, W., Tarlinton, D.M., and Nutt, S.L. (2017). Environmental sensing by mature B cells is controlled by the transcription factors PU.1 and SpiB. *Nature Communications* 8, 1426.
- Wirth, A., Jung, M., Bies, C., Frien, M., Tyedmers, J., Zimmermann, R., and Wagner, R. (2003). The Sec61p Complex Is a Dynamic Precursor Activated Channel. *Molecular Cell* 12, 261-268.
- Wöhner, M., Tagoh, H., Bilic, I., Jaritz, M., Poliakova, D.K., Fischer, M., and Busslinger, M. (2016). Molecular functions of the transcription factors E2A and E2-2 in controlling germinal center B cell and plasma cell development. *Journal of Experimental Medicine* 213, 1201-1221.
- Wudhikarn, K., Wills, B., and Lesokhin, A.M. (2020). Monoclonal antibodies in multiple myeloma: Current and emerging targets and mechanisms of action. *Best Practice & Research Clinical Haematology* 33, 101143.
- Xia, D., Tang, W.K., and Ye, Y. (2016). Structure and function of the AAA+ ATPase p97/Cdc48p. *Gene* 583, 64-77.
- Xia, Y., Xu-Monette, Z.Y., Tzankov, A., Li, X., Manyam, G.C., Murty, V., Bhagat, G., Zhang, S., Pasqualucci, L., Visco, C., *et al.* (2017). Loss of PRDM1/BLIMP-1 function contributes to poor prognosis of activated B-cell-like diffuse large B-cell lymphoma. *Leukemia* 31, 625-636.
- Xu, H., Chaudhri, V.K., Wu, Z., Biliouris, K., Dienger-Stambaugh, K., Rochman, Y., and Singh, H. (2015). Regulation of bifurcating B cell trajectories by mutual antagonism between transcription factors IRF4 and IRF8. *Nature Immunology* 16, 1274-1281.

- Yamaguchi, D., Hu, D., Matsumoto, N., and Yamamoto, K. (2009). Human XTP3-B binds to α 1-antitrypsin variant nullHong Kong via the C-terminal MRH domain in a glycan-dependent manner. *Glycobiology* 20, 348-355.
- Yamamoto, K., Sato, T., Matsui, T., Sato, M., Okada, T., Yoshida, H., Harada, A., and Mori, K. (2007). Transcriptional Induction of Mammalian ER Quality Control Proteins Is Mediated by Single or Combined Action of ATF6 α and XBP1. *Developmental Cell* 13, 365-376.
- Ye, B.H., Cattoretti, G., Shen, Q., Zhang, J., Hawe, N., Waard, R.d., Leung, C., Nouri-Shirazi, M., Orazi, A., Chaganti, R.S.K., *et al.* (1997). The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. *Nature Genetics* 16, 161-170.
- Ye, J., Rawson, R.B., Komuro, R., Chen, X., Davé, U.P., Prywes, R., Brown, M.S., and Goldstein, J.L. (2000). ER Stress Induces Cleavage of Membrane-Bound ATF6 by the Same Proteases that Process SREBPs. *Molecular Cell* 6, 1355-1364.
- Ye, Y., Meyer, H.H., and Rapoport, T.A. (2001). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature* 414, 652-656.
- Yoshida, H., Matsui, T., Hosokawa, N., Kaufman, R.J., Nagata, K., and Mori, K. (2003). A Time-Dependent Phase Shift in the Mammalian Unfolded Protein Response. *Developmental Cell* 4, 265-271.
- Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001). XBP1 mRNA Is Induced by ATF6 and Spliced by IRE1 in Response to ER Stress to Produce a Highly Active Transcription Factor. *Cell* 107, 881-891.
- Yu, B., Jiang, T., and Liu, D. (2020). BCMA-targeted immunotherapy for multiple myeloma. *J Hematol Oncol* 13, 125.
- Yu, D., Rao, S., Tsai, L.M., Lee, S.K., He, Y., Sutcliffe, E.L., Srivastava, M., Linterman, M., Zheng, L., Simpson, N., *et al.* (2009). The Transcriptional Repressor Bcl-6 Directs T Follicular Helper Cell Lineage Commitment. *Immunity* 31, 457-468.
- Zhang, K., Wong, H.N., Song, B., Miller, C.N., Scheuner, D., and Kaufman, R.J. (2005). The unfolded protein response sensor IRE1 α is required at 2 distinct steps in B cell lymphopoiesis. *The Journal of clinical investigation* 115, 268-281.
- Zhang, R., Alt, F.W., Davidson, L., Orkin, S.H., and Swat, W. (1995). Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the *vav* proto-oncogene. *Nature* 374, 470-473.
- Zhang, T., Wong, S.H., Tang, B.L., Xu, Y., Peter, F., Subramaniam, V.N., and Hong, W. (1997). The mammalian protein (rbet1) homologous to yeast Bet1p is primarily associated with the pre-Golgi intermediate compartment and is involved in vesicular transport from the endoplasmic reticulum to the Golgi apparatus. *J Cell Biol* 139, 1157-1168.

Zhou, J.H., Markham, J.F., Duffy, K.R., and Hodgkin, P.D. (2018). Stochastically Timed Competition Between Division and Differentiation Fates Regulates the Transition From B Lymphoblast to Plasma Cell. *Frontiers in immunology* 9, 2053.

Zipfel, P.A., Grove, M., Blackburn, K., Fujimoto, M., Tedder, T.F., and Pendergast, A.M. (2000). The c-Abl tyrosine kinase is regulated downstream of the B cell antigen receptor and interacts with CD19. *J Immunol* 165, 6872-6879.