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To my family

"One never notices what has been done;
one can only see what remains to be done."

Maria Skłodowska- Curie

ABSTRACT

Paulina Budzyńska

Regulation of B cell development by transcription factors IRF4 and BACH2

The University of Turku, Faculty of Medicine, Institute of Biomedicine, Department of Medical Microbiology and Immunology, Turku Doctoral Programme of Molecular Medicine (TuDMM) and, Turku Doctoral Programme of Biomedical Sciences (TuBS) Turku, Finland

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B lymphocytes—major cell types mediating an adaptive immunity—recognize a vast variety of structures by B cell antigen receptors (BCR). Activated B cells can form germinal centers, undergo antigen-induced affinity maturation of their BCRs and differentiate into memory B cells sought upon vaccinations or antibody-secreting plasma cells that eradicate infections. Aberrant BCR signaling or transcriptional regulation of B cell development may cause immunodeficiencies, autoimmune diseases or lymphoid malignancies. Therefore, deeper knowledge of the transcriptional regulation of B cell development is essential for the understanding of B cell derived diseases.

Transcription factors IRF4 and BACH2 are crucial regulators of germinal center formation and high-affinity antibody production. However, the molecular mechanisms how they do these remain unknown. The aim of this study was to investigate the role of IRF4 in B cells and clarify IRF4 function in the regulation of antibody secretion as well as to examine the role of BACH2 in immunoglobulin (Ig) diversification processes. To do this IRF4-, BACH2-, and IRF4/BCL6 double-deficient DT40 B cell lines were generated.

In this thesis, I demonstrate a new role for IRF4 as a regulator of BCR signaling, which is crucial for the B cell survival, and activation. IRF4 directly represses *IRF8* and upregulates *SHIP* gene expression that results in stronger BCR-induced calcium flux and changed signaling to ERK and PI3K/AKT signaling pathways. In this study, I show nonredundant functions for IRF4 and BLIMP1 in the induction of antibody secretion, as in IRF4- or IRF4/BCL6 double-deficient cells enforced expression of BLIMP1 fails to induce antibody secretion. As the most important finding, I reveal a new role for BACH2 in Ig gene conversion that diversifies BCR.

Keywords: adaptive immunity, B cells, plasma cells, antibody diversification, gene conversion, transcription factors, IRF4, BACH2, BLIMP1, AID

TIIVISTELMÄ

Paulina Budzyńska

Transkriptiotekijät IRF4 ja BACH2 B-solujen kehityksen säätelijöinä

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteellinen mikrobiologia ja immunologia, Turun molekyylilääketieteen tohtoriohjelma (TuDMM) ja Turun biolääketieteellinen tohtoriohjelma (TuBS), Turku, Suomi

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B-solut ovat hankitun immuunijärjestelmän soluja, jotka tunnistavat valtavan määrän erilaisia rakenteita B-solujen antigeenireseptorilla. Aktivoidut B-solut voivat muodostaa itukeskuksia, käydä läpi B-solureseptorin affiniteetin kypsymisen ja erilaistua muisti-B-soluiksi, johon rokottamisellakin pyritään, tai erilaistua vasta-aineita tuottaviksi plasmasoluiksi infektioiden hävittämiseksi. Virheelliset signaalit B-solureseptorista tai virheellinen B-solukehityksen säätely voivat aiheuttaa immuunipuutoksia, autoimmuunisairauksia tai B-solusyöpiä. Tästä syystä perusteellinen B-solukehityksen ja sen säätelyn tunteminen transkription tasolla on välttämätöntä B-solusairauksien ymmärtämiseksi.

Transkriptiotekijät IRF4 ja BACH2 ovat välttämättömiä itukeskuksen muodostumiselle ja voimakas affiniteettisten vasta-aineiden synnylle. Näiden ilmiöiden taustalla olevia molekyylitason mekanismeja ei tunneta. Tämän tutkimuksen tavoitteena oli selvittää IRF4:n suoria kohdegeenejä ja valottaa sen toimintaa vasta-aineiden erittämisen säätelijänä, sekä tutkia BACH2:n osuutta vasta-aineiden monimuotoistumisessa. Tätä varten tehtiin IRF4- ja BACH2-poistogeeniset DT40 B-solulinjat sekä IRF4/BCL6-kaksoispoistogeeninen DT40 B-solulinja.

Tässä väitöskirjassa osoitan, että IRF4:llä on tärkeä osuus B-solujen antigeenireseptorin signaalinvälityksen säätelijänä. IRF4 estää IRF8:n ja SHIP:n tuotantoa, mikä lisää sytoplasman kalsium-ionien määrää ja muuttaa ERK- ja PI3K/AKT-signaalinvälitysteiden aktiivisuutta. Tässä työssä näytän myös että IRF4:llä ja BLIMP1:llä on erilliset tehtävät vasta-aineen erityksen käynnistämisessä, sillä pakotettu BLIMP-1:n ilmentäminen IRF4- tai IRF4/BCL6-postogeenisissä soluissa ei käynnistä eritystä. Tärkeimpänä löydöksenä osoitan, että BACH2 lisää B-solureseptoria monimuotoistuvaa geenikonversiota.

Avainsanat: Hankittu immuniteetti, B-solut, plasmasolut, vasta-aineiden monimuotoistuminen, transkriptiotekijät, IRF4, BACH2, BLIMP1, AID

TABLE OF CONTENTS

ABBREVIATIONS

AEJ	Alternative end joining
AICE	AP-1-IRF composite motifs
AICDA	Activation-induced cytidine deaminase (gene)
AID	Activation-induced cytidine deaminase (protein)
AKT	AKT Serine/Threonine kinase 1
ALV	Avian Leucosis Virus
BACH2	BTB and CNC homology 2
BACH2KO	BACH2 knockout DT40 cell line
BCAP	B cell Adaptor Protein
BCL6	B cell lymphoma 6
BCL6KO	BCL6 knockout DT40 cell line
BCR	B cell antigen receptor
BER	Base excision repair
BLIMP1	B lymphocyte-induced maturation protein
BLNK	B cell linker protein
BTB	Bric à brac, tramtrack, broad-complex
BTK	Bruton’s Tyrosine Kinase
bZip	basic leucine zipper
C	Constant
CD	Cluster of differentiation
ChIP	Chromatin Immunoprecipitation
CNC	Cap'n'collar
CSR	Class-switch recombination
D	Diversity
DKO	IRF4 and BACH2 double knockout DT40 cell line
DZ	Dark zone
ERK	Extracellular signal-regulated kinase
FDC	Follicular dendritic cell
FO	Follicular
GC	Germinal center
GCV	Immunoglobulin gene conversion
GEO	Gene Expression Omnibus
HSC	Hematopoietic Stem Cell
MARE	MAF recognition element
MHC II	Class II Major Histocompability Complex

MMR	Mismatch repair
MZ	Marginal zone
NF-κB	Nuclear Factor Kappa B
NHEJ	Non-homologous end joining
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IgV	Immunoglobulin variable region
IL	Interleukin
IP	Immunoprecipitation
IRF4	Interferon Regulatory Factor 4
IRF4KO	IRF4 knockout DT40 cell line
IRF8	Interferon Regulatory Factor 8
ISRE	Interferon sequence response elements
ITAM	Immunoreceptor Tyrosine-based Activation Motif
J	Joining
LPS	Lipopolysaccharide
LZ	Light zone
MAPK	Mitogen-Activated Protein Kinase
PAX5	Paired box protein 5
PI3K	Phosphatidylinositol 3-kinase
PLCγ2	Phospholipase Cγ2
PRDM1	Positive regulatory domain containing 1
POZ	Pox virus and zinc finger
RAG	Recombination activating gene
RIPA	Radioimmunoprecipitation assay buffer
SHIP	SH2-containing inositol phosphatase
SHM	Somatic hypermutation
SYK	Spleen tyrosine kinase
T_{FH}	Follicular helper T cell
TIRF	Total internal reflection fluorescence
UNG	Uracil DNA glycosylase
UPR	Unfolded protein response
V	Variable
XBP1	X-box binding protein 1

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by Roman numerals (I-III):

I Budzyńska, P.M., Niemelä, M., Sarapulov, A.V., Kyläniemi, M.K., Nera, K.P., Junttila, S., Laiho, A., Mattila, P.K., Alinikula, J., and Lassila, O. (2015). IRF4 deficiency leads to altered BCR signaling revealed by enhanced PI3K pathway, decreased SHIP expression and defected cytoskeletal responses. *Scand. J. Immunol*. 82:418-28.

II Budzyńska, P.M., Kyläniemi, M.K., Alinikula, J., Lassila, O. and Nera, K.P. BLIMP1 is insufficient to induce antibody secretion in the absence of IRF4. *Manuscript*

III Budzyńska, P.M., Kyläniemi, M.K., Kallonen, T., Soikkeli, A.I., Nera, K.P., Lassila, O., and Alinikula J. (2017). Bach2 regulates AID-mediated immunoglobulin gene conversion and somatic hypermutation in DT40 B cells. *Eur. J. Immunol.* 47:993-1001.

1 INTRODUCTION

Vertebrates defend themselves against foreign invaders with the help of innate and adaptive immunity. Innate immunity provides the first line of defense. It prevents the entrance of microbes by anatomical barriers (epithelial barrier) for example skin and rapidly eliminates successful invaders by natural killer cells, phagocytes, and the complement system. The adaptive immunity response develops later, after the entrance of microbe to the tissue and is mediated by B and T lymphocytes and their products (for example antibodies and cytokines). Pathogen-specific neutralizing antibodies are the most desirable products of the adaptive immune system. Antibodies are a soluble form of B cell antigen receptors (BCR) secreted by plasma cells, which are terminally differentiated B lymphocytes (also called B cells). The development of B cells into high-affinity antibody secreting plasma cells is composed of multiple differentiation stages, each of which is tightly regulated by several transcription factors. In early stages of B cell development, B cells assemble their primary repertoire of B cell antigen receptors via immunoglobulin gene rearrangements [V(D)J recombination] complemented, in some species, by immunoglobulin gene conversions (GCV). This primary repertoire of a vast number of BCRs uniquely expressed on each B lymphocyte is produced in an antigen-independent manner. A selection driven by the strength of pre-BCR and BCR signaling eradicates non-functional or self-reactive B lymphocytes. B cell fate decisions including proliferation, survival, activation and development critically depend on the BCR signaling. Therefore, BCR signaling machinery requires strict control by transcription factors as dysregulation of BCR signaling may lead to aberrant B cell development, function, and immunodeficiency such as Bruton's agammaglobulinemia (Bruton, 1952).

Late B cell development initiates the response of mature B cell to foreign antigen. Antigen-activated B cells can differentiate into short-lived plasmablasts or form the germinal center (GC), a histological structure in secondary lymphoid organs, where B cell affinity maturation occurs and from where memory B cells and long-lived plasma cells arise sought in severe infections and effective vaccinations. Germinal center B cells obtain antigen-specific, high-affinity BCRs by combined outcome of rapid cell proliferation, high rate of somatic hypermutation in immunoglobulin genes (SHM), and selection of best antigen binders with simultaneous elimination of autoreactive or deleterious mutation-carrying B cells. The fact that most human lymphomas arise in germinal centers, malfunction of the regulatory system of B cells may have severe consequences in the form of autoimmune diseases and development of lymphoid malignancies.

Transcription factors control the B cell development and function by coordinated adjustments in the expression of thousands of genes involved in B cell differentiation. Of great interest is to understand how humoral immunity constituted entirely by B cells works. Therefore, thorough studies of transcription factors and their target genes underlying the cell fate decisions and effector function of B cells are needed. This basic knowledge is important for understanding B cell derived medical problems like leukemias, lymphomas, and immunodeficient patients, and for the development of efficient vaccinations that would stimulate the production of high-affinity antibodies neutralizing pathogens such as HIV.

The focus of this thesis is on understanding the role of transcription factors Interferon Regulatory Factor 4 (IRF4) and Bric à brac, tramtrack, broad-complex (BTB) and the cap'n'collar (CNC) homology 2 (BACH2) in B cell development. In the absence of these factors immunized mice cannot form germinal centers and produce high-affinity antigen-specific antibodies. Genetic studies in mouse system have revealed that graded expression of IRF4 regulates B cell development and is critical for plasma cell differentiation. However, the mechanism, how IRF4 performs its crucial role, is largely unknown and the role of IRF4 in antibody secretion remains unclear since conditional inactivation of IRF4 in mouse plasma cells causes immediate cell death. Therefore, I studied the role of IRF4 in B cells and in antibody secretion in the immortalized chicken DT40 B cell line by gene targeting approach. Whereas stimulated mouse *Irf4*$^{-/-}$ B cells cannot become antibody-secreting plasma cells, the *Bach2*$^{-/-}$ B cells rapidly differentiate into plasma cells secreting of low-affinity IgM antibodies indicating that BACH2 is involved in B cell affinity maturation process. In this thesis, the role of BACH2 in the generation of mutations in immunoglobulin light chain locus is examined by deletion of *BACH2* in DT40 B cell line which is commonly used for studies of immunoglobulin diversification processes such as gene conversion (GCV) and somatic hypermutation (SHM) that also take place in germinal center B-cell affinity maturation.

2 REVIEW OF LITERATURE

2.1 Generation of primary B cell repertoire

B cells produce a highly diversified repertoire of antibodies against fast-evolving pathogens. In humans and mice, much of this diversity is achieved by a random assembly of antibody genes from an extensive collection of the individual variable (V), diversity (D) and joining (J) gene segments (Hozumi and Tonegawa, 1976). However, in many species, (e.g. chicken, rabbit, and horse) V(D)J recombination is inefficient in the production of an extensive repertoire of antigen receptors due to a very low number of functional V and J segments. Therefore V(D)J recombination serves only for assembly of functional genes from immunoglobulin (Ig) segments in these species, and the diversity in rearranged V(D)J segments is primarily formed by Ig gene conversion (Reynaud et al., 1987). In this process patches of many different pseudo V genes are copied into the functional IgV region by the homologous recombination-like process (Weill and Reynaud, 1987).

2.1.1 V(D)J recombination defines early B cell developmental stages in mammals

B cells undergo multistage development to achieve high-affinity B cell antigen receptors (BCR). When secreted they are known as antibodies. B cells arise from hematopoietic stem cells, a self-renewing reservoir of progenitors for all blood cells (Orkin and Zon, 2008).

The early developmental stages of B cells (in mammals) occur in bone marrow and are defined by subsequent rearrangement of Ig genes, also known as V(D)J recombination (Figure 1). Each of the immunoglobulin V, D and J segments is flanked by recombination signal sequences. These sequences are recognized, brought together and cleaved by the recombination activating enzymes (RAG1 and RAG2) in a complex with additional proteins (Oettinger et al., 1990). Arising double–strand DNA breaks between appropriate coding segments are then ligated via non-homologous end joining (NHEJ) machinery (Hendrickson et al., 1991) resulting in the recombined V-J or V-D-J gene lacking intervening DNA sequence.

The rearrangement of the Ig heavy (H) chain locus defines the pro-B cell stage, where D_H segments are randomly combined with J_H segments and then V_H seg-

ments with already recombined DJ_H segments (Alt et al., 1984; Early et al., 1980). This combinatorial diversity of V, D and J gene segments is increased by junctional diversity created by deletion or addition of base pairs at the junction of gene segments. Terminal deoxynucleotidyl transferase, an enzyme catalyzing the addition of random, non-templated base pairs has a major role in creation of junctional diversity (Desiderio et al., 1984). After recombination of IgH chain locus, the μ heavy chain protein is expressed, mostly in the cytoplasm but some of it together with surrogate light chain composed of λ5 and V pre-B and signal transducing components Igα and Igβ (encoded by *CD79A* and *CD79B*, respectively) form pre-B cell receptor (pre-BCR) on the surface of large pre-B cell (Brouns et al., 1995; Karasuyama et al., 1990; Kerr et al., 1989; Tsubata and Reth, 1990; Winkler et al., 1995). Pre-B cells failing to express pre-BCR undergo apoptosis as signaling through pre-BCR is required for proliferation and further development into small pre-B cells (Herzog et al., 2009; Kline et al., 1998).

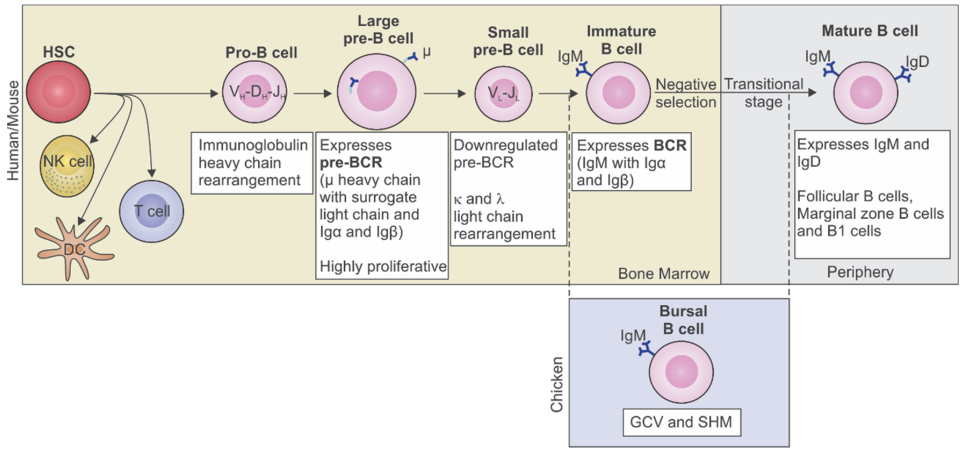

Figure 1. B cell developmental stages
Development of hematopoietic stem cell (HSC) toward mature B cell is defined by the steps of immunoglobulin (Ig) rearrangements. The alternative avian B-cell developmental stage is indicated. NK, natural killer cell; DC, dendritic cell; BCR, B cell antigen receptor; GCV, gene conversion; SHM, somatic hypermutation.

Small pre-B cells are withdrawn from proliferation and downregulate expression of pre-BCR allowing rearrangement of light (L) chain V_L-J_L gene segments. Pre-B cells start their light chain rearrangement first with the Igκ locus and then Igλ. Whichever produce functional light chain associates with recombined μ heavy chain and Igα and Igβ to form a complete IgM antigen receptor on the surface of the immature B cell. This newly composed BCR carried by each lymphocyte is tested for its specificity and reactivity toward self-antigens. Self-reactive B cells undergo apoptosis or additional round of IgL rearrangement (Goodnow et al., 1988; Hartley et al., 1991) and self-tolerant B cells can leave the bone marrow and travel to the spleen. Before the fully mature stage, B cells pass through early

and late transitional stages (T1, T2, and T3) defined by their surface marker expression. The mature B cells are distinguished from immature B cells by the simultaneous expression on the surface IgM and IgD (a spliced variant of IgH) (Figure 1).

2.1.2 Chicken B cell development

The avian (chicken) early B cell development is to some extent similar to that observed in the mammals (human and mice) (Nera, 2015). Chicken hematopoiesis starts in intraembryonic regions (Lassila et al., 1978) and at the pre-bursal stage, avian B cell progenitors begin their immunoglobulin V_H and V_L genes rearrangement (Mansikka et al., 1990). In chicken, the heavy chain recombination does not always precede the light chain recombination as an opposite order is observed frequently (Benatar et al., 1992). After pre-bursal stage surface IgM^+ B cells migrate to bursa of Fabricius (Lassila et al., 1979). In the bursal lymphoid follicles B cell repertoire formation take place by immunoglobulin gene conversion (GCV) with a low frequency of somatic hypermutation (a single nucleotide somatic untemplated mutations in IgV) (Arakawa et al., 2002b) (Figure 1). Several months after birth the bursa of Fabricius involutes and loses its primary B cell repertoire formation function (Arakawa and Buerstedde, 2004). In contrast, human and mice continuously generate their B cell repertoire in bone marrow. The bursal stage is not present in mice and human, but the post-bursal stages are very similar to those observed in mammals. Upon antigen encounter chicken B cells can form germinal centers in which they undergo further immunoglobulin gene diversification by high rates of somatic hypermutation and low frequency of GCV (Arakawa and Buerstedde, 2004; Nera, 2015).

2.1.3 Immunoglobulin gene conversion (GCV)

The gene conversion was first discovered in chicken (Reynaud et al., 1987) but is not an exclusive avian process. Many other species such as rabbits (Becker and Knight, 1990), cattle, swine, horses (Butler, 1998), guinea pigs (Guo et al., 2012), Tasmanian devils (Qin et al., 2015) and prairie voles (Ujvari and Belov, 2015) have been shown to use it for primary B cell repertoire formation. It seems that most mammals and avian species compose the primary B cell repertoire via gene conversion suggesting that this mechanism evolved long time ago and was lost during evolution process to mice, rats and human (Arakawa and Buerstedde, 2004).

The gene conversion mostly appears in the appendix in rabbits (Becker and Knight, 1990), the bursa of Fabricius in chicken (Reynaud et al., 1987) and the ileal Peyer's patches in, swine, cattle, and horses (Butler, 1998).

2.1.3.1 GCV in chicken

In chicken, non-functional pseudogenes: 25 pseudo-Vs (ψV) for IgL (Reynaud et al., 1987) and approx. 80 pseudo V-Ds for IgH (Reynaud et al., 1989) are located upstream of functional V region of IgL ($V_{\lambda 1}$) and IgH (V_{H1}) loci. These pseudogenes are more conserved than the single functional V gene (McCormack et al., 1993) and possess neither promoter nor leader exon and V(D)J recombination signal sequences (Arakawa and Buerstedde, 2004).

During gene conversion fragments (8-200 bp) of the ψVs are copied into functional V gene without loss or change of donor sequence (McCormack and Thompson, 1990; Reynaud et al., 1987) (Figure 2). Gene conversion occurs only between homologous ψV genes and rearranged V (D) J gene located on the same chromosome (Carlson et al., 1990). Since gene conversion is a homologous recombination-like process, the ψV gene either more homologous, closer or in the opposite orientation to the acceptor V gene sequence is more frequently used (McCormack and Thompson, 1990; Sayegh et al., 1999). Copied sequence of one pseudo V into rearranged V gene creates more homology to another pseudo-V gene leading to a stepwise editing of the IgV locus (Arakawa et al., 1996). A 5' to 3' polarity in the copying of the gene conversion tracks seems to occur. The 5' end of the pasted sequence is always homologous to the recipient V region, whereas the 3' end does not have to be homologous and often encompasses insertions or deletions of nucleotides (McCormack and Thompson, 1990). Due to the flexibility of pseudo-V gene assembly, the gene conversion is predicted to diversify the Ig gene more than V(D)J recombination (Arakawa and Buerstedde, 2004).

2.1.3.2 GCV in chicken DT40 B cell line

Important knowledge about the molecular mechanism of gene conversion and transcriptional regulation of B cell development comes from studies conducted in the chicken bursal B cell line DT40 (Alinikula et al., 2006; Nera, 2015). This avian leucosis virus (ALV)-induced lymphoma line DT40 (Baba et al., 1985), similarly to bursa cells (from which originates), continuously undergoes IgL gene conversion accompanied by somatic hypermutation (Buerstedde et al., 1990).

The surface IgM (sIgM) deficient variant of DT40 B cell line (called Clone 18) carries a frameshift mutation in the rearranged Ig light chain V segment that can be repaired by overlapping gene conversion/somatic hypermutation events leading to the re-expression of sIgM (Buerstedde et al., 1990). This property is commonly used for estimation of gene conversion frequency in the sIgM gain assay (see more in *Materials and methods* section). Furthermore, transfected DNA constructs are integrated (targeted integration) at high ratios into endogenous loci (Buerstedde and Takeda, 1991). This characteristic makes DT40 cell line as an excellent tool for studying transcriptional regulation in B cells by loss of function experiments (Takeda et al., 1992).

2.1.3.3 Overview of molecular mechanism of GCV, somatic hypermutation (SHM) and class-switch recombination (CSR)

The activation-induced cytidine deaminase (AID) is a DNA modifying enzyme critical for GCV, somatic hypermutation (SHM) and class-switch recombination (CSR) since AID disruption completely blocks these processes (Arakawa et al., 2002a; Harris et al., 2002; Muramatsu et al., 2000; Revy et al., 2000). AID catalyzes the deamination of cytidine in a single-stranded DNA (mostly in Ig loci) yielding uracil in DNA (Di Noia and Neuberger, 2007; Petersen-Mahrt et al., 2002). The AID-induced DNA lesions in Ig can lead to different outcomes: GCV, SHM or CSR (a change of antibody effector function) (Figure 2).

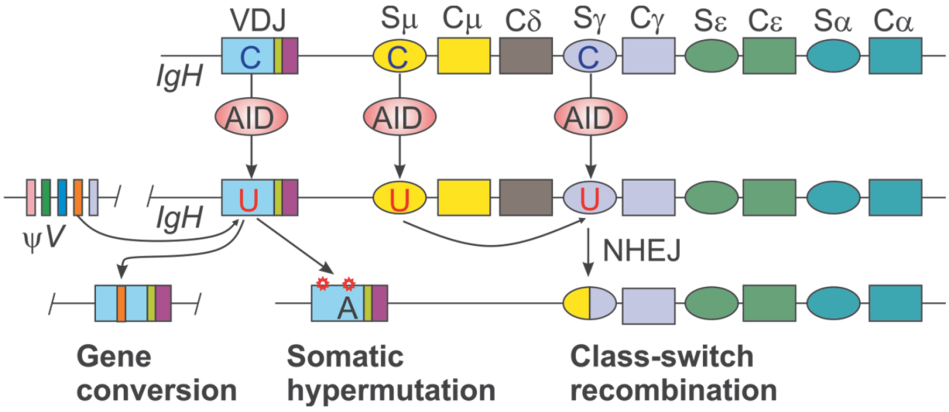

Figure 2. AID-induced immunoglobulin gene diversification processes
AID catalyzes the deamination of cytidine (C) into uracil (U) which can be processed by homologous recombination, non-templated single base substitution or non-homologous end joining (NHEJ) resulting in gene conversion, somatic hypermutation, and class-switch recombination, respectively. V, variable; D, diversity; J, joining; C, constant; S, switch regions of functional immunoglobulin genes. Modified from (Harris and Liddament, 2004).

The outcome depends on the exact location of the cytidine deamination in the functional Ig gene and the type of DNA repair pathway used for elimination of uracil from DNA (Neuberger et al., 2003). The presence of uracil in DNA is causing U:G mispairing (Lindahl, 1993) which if left unrepaired may lead to mutations of C to T and G to A in the next round of replication and mutant proteins (Duncan and Weiss, 1982; Impellizzeri et al., 1991; Viswanathan et al., 1999). Therefore, the presence of uracil in DNA involves multiple DNA repair pathways, including: a mismatch repair (MMR) pathway (with Msh2/Msh6 heterodimer complex participating in the process) (Roa et al., 2010) or, most commonly, a base excision repair pathway (BER) (Lindahl, 2000) involving uracil DNA glycosylase (UNG). Removal of uracil from DNA by UNG results either in a single-strand DNA break or a double-strand DNA break (Di Noia and Neuberger, 2002; Rada et al., 2002). These DNA breaks occurring in the IgV region are intermediate for GCV and as such are sufficient to induce GCV (Bastianello and Arakawa, 2017). The DNA breaks in the IgV locus in the presence of ψVs are predominantly repaired in a recombination-mediated manner engaging homologous recombination (HR) pathway and to a lesser extent are maintained by translesion DNA synthesis (TLS) or NHEJ pathways. However, in the absence of ψVs, the situation is opposite, and repair of uracil may result in an SHM event (Arakawa et al., 2004). Factors which have a role in DNA repair (BER, MMR, HR, NHEJ, TLS, and AEJ) or transcriptional regulation and whose role in the Ig GCV was revealed by gene disruption in DT40 cell line are listed in Table 1. Genes which facilitate GCV, promote GCV and prevent SHM, or repress GCV are among these factors.

Table 1. Ig gene conversion studies conducted in DT40 mutants

Gene knockout	General function/ property	DT40 mutant phenotype	References
AICDA (Activation-induced cytidine deaminase) encoding AID	Cytidine deaminase	No GCV[a] activity and SHM[b]	(Arakawa et al., 2002a; Harris et al., 2002)
UNG (Uracil DNA glycosylase**)**	Uracil DNA glycosylase, BER[c] and AEJ[d]	Dramatically reduced GCV[a] with high rate of SHM[b]	(Saribasak et al., 2006)
ψV genes (pseudo variable genes)	Donor sequence for Ig gene conversion	Shift from GCV[a] to SHM[b]	(Arakawa et al., 2004)
RAD52 (RAD52 homolog, DNA repair protein)	HR[e]	No change in GCV[a] and slight reduction of targeted integration	(Yamaguchi-Iwai et al., 1998)
RAD54 (RAD54-like)	HR[e]	Reduction of GCV[a] and targeted integration	(Bezzubova et al., 1997)
NBS1 (NBN, nibrin)	HR[e]	Reduction of GCV[a] and targeted integration	(Tauchi et al., 2002)
RAD51 recombinase paralogues: ***XRCC2*** and ***XRCC3*** (X-ray repair cross complementing 2 and 3), and ***RAD51B*** (RAD51 paralog B)	HR[e]	Shift from GCV[a] to SHM[b]	(Sale et al., 2001)

Gene knockout	General function/ property	DT40 mutant phenotype	References
BRCA1 or ***BRCA2*** (DNA repair associated)	HR[e]	Shift from GCV[a] to SHM[b]	(Hatanaka et al., 2005; Longerich et al., 2008)
FANCD2 or ***FANCC*** (Fanconi anemia complementation group D2 or C)	HR[e]	Reduction of GCV[a], SHM[b] (and targeted integration in FANCD2 mutant)	(Niedzwiedz et al., 2004; Yamamoto et al., 2005)
RAD9 (Checkpoint clamp component) or ***RAD17*** (Checkpoint clamp loader component)	HR[e]	Reduced GCV[a] with high rates of SHM[b]	(Saberi et al., 2008)
RAD18 (E3 ubiquitin protein ligase)	HR[e]	Reduction of GCV[a]	(Szuts et al., 2006)
MSH6 (MutS homolog 6) or ***PMS2*** (PMS1 homolog 2, mismatch repair system component)	MMR[f]	Cell cycle defect and slight effect on GCV[a] activity	(Campo et al., 2013)
PRKDC (protein kinase, DNA-activated, catalytic polypeptide) or ***XRCC6*** (**KU70**) (X-ray repair complementing defective repair in Chinese hamster cells 6)	NHEJ[g]	Increased GCV[a]	(Tang and Martin, 2006)
PARP1 (poly (ADP-ribose) polymerase 1) or ***FEN1*** (flap structure-specific endonuclease 1)	BER[c] and AEJ[d]	Reduced GCV[a]	(Kikuchi et al., 2005; Paddock et al., 2010)
PARP1/KU70 **double-deficient**	BER[c] and NHEJ[d]	No effect on GCV[a] activity	(Paddock et al., 2011)
REV1 (DNA directed polymerase)	TLS[h]	Decreased GCV[a], chromosomal instability/ fragility; reduction of SHM[b]	(Okada et al., 2005; Simpson and Sale, 2003)
REV3L (REV3 like, DNA directed polymerase zeta catalytic subunit)	TLS[h]	No effect on GCV[a], reduced targeted integration, hypersensitivity to various genotoxic treatments	(Okada et al., 2005)
POLH (DNA polymerase eta)	TLS[h]	Slight decrease in GCV[a]	(Hirota et al., 2010)
POLQ or ***POLN*** (DNA polymerase theta or nu)	TLS[h]	No effect on GCV[a]	(Kohzaki et al., 2010)
POLN**/*****POLQ*** **double-deficient**	TLS[h]	Decreased GCV[a]	(Kohzaki et al., 2010)
POLH**/*****POLN*****/*****POLQ*** **triple-deficient**	TLS[h]	No GCV[a] activity and reduced SHM[b]	(Kohzaki et al., 2010)
ASCIZ (ASCIZ zinc finger protein)	DNA damage response	Increased GCV[a]	(Oka et al., 2008)
HDAC2 (histone deacetylase 2)	Histone deacetylase	Enhanced GCV[a]	(Lin et al., 2008)
BAP1 (BRCA1 associated protein 1)	Deubiquitinase of histone H2A	Reduction of GCV[a]	(Yu et al., 2014)
TOP1 (topoisomerase (DNA) I) **knockdown**	Topoisomerase	Increased GCV[a] and SHM[b] with higher Pol II density and recruitment of AID to IgV locus	(Maul et al., 2015)
BLM (Bloom syndrome RecQ like helicase)	DNA helicase	Reduction of GCV[a] and targeted integration	(Kikuchi et al., 2009)
RECQL5 (RecQ like helicase 5)	DNA helicase	Increased GCV[a]	(Hosono et al., 2014)

Gene knockout	General function/ property	DT40 mutant phenotype	References
MCM3AP **(GANP)** (minichromosome maintenance complex component 3 associated protein) **haplodeficient**	Interacts with AID, involved in DNA repair	Reduction of GCV[a] and SHM[b]	(Eid et al., 2014)
IKZF3 (IKAROS family zinc finger 3) encoding AIOLOS	Transcription factor	Impaired GCV[a]	(Narvi et al., 2007)
TCF3 (transcription factor 3) encoding E2A	Transcription factor	Strongly reduced SHM[b], GCV[a], and histone H4 acetylation in IgL locus	(Kitao et al., 2008; Schoetz et al., 2006)
BCL6 (B cell lymphoma 6)	Transcription factor	No GCV[a] and SHM[b] activity	(Williams et al., 2016)
NFKB1 **(p-50)** (nuclear factor kappa B subunit 1) or ***REL*** **(c-Rel)** (REL proto-oncogene, NF-kB subunit)	NF-κB family transcription factors	Reduction of GCV[a]	(Kim and Tian, 2009)
SUB1 **(PC4)** (SUB1 homolog, transcriptional regulator)	Single-stranded DNA binding protein with a role in DNA repair	Decreased GCV[a] and SHM[b] with no effect on targeted integration	(Caldwell et al., 2016)

[a)] GCV, immunoglobulin gene conversion; [b)] SHM, somatic hypermutation; [c)] BER, base excision repair; [d)] AEJ, alternative end joining; [e)] HR, homologous recombination; [f)] MMR, mismatch repair; [g)] NHEJ, non-homologous end joining; [h)] TLS, translesion DNA synthesis

2.2 Late B cell development

All mature B cells residing in the periphery upon antigen activation are capable of rapid proliferation, class-switch recombination (CSR) and differentiation into short-lived plasmablasts (secreting mostly IgM) (Figure 3). In most cases, this rapid response occurs for nonprotein antigens (e.g. bacterial polysaccharides and lipids) without T cell help (T-independent activation). On the contrary, B cells require T cell help (T-dependent activation) for activation with protein/peptide antigens and initiation of immediate and persistent protection. Upon such support, B cells can differentiate into short-lived plasmablasts and also form germinal centers, histological structures where affinity maturation of B cell antigen receptor occurs (Figure 3). The germinal center (GC) B cells give rise to long-lived plasma cells (secreting antibodies of different classes) or memory B cells that provide immunological memory sought after vaccinations and viral or bacterial infections. Some CSR also occurs without GCs, but no SHM.

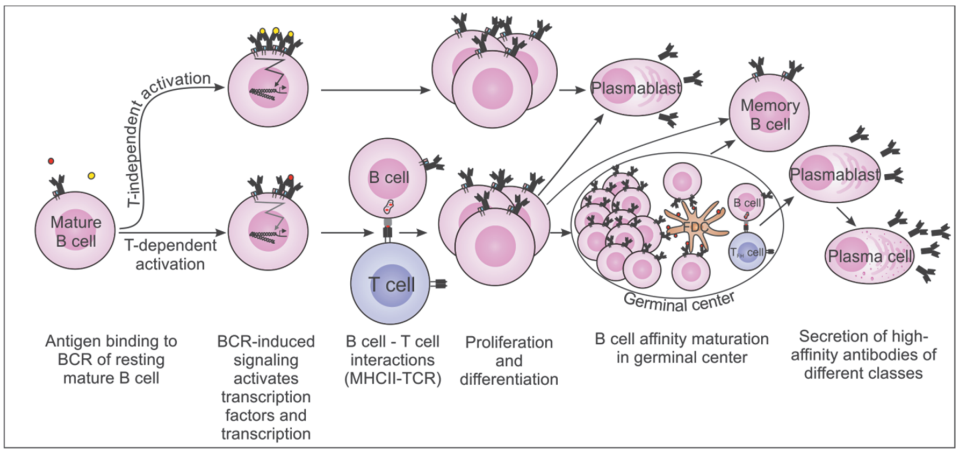

Figure 3. The differentiation of mature B cells upon antigen encounter
The red circle represents protein or peptide whereas yellow circles nonprotein antigens. FDC, follicular dendritic cell; BCR, B cell antigen receptor; TCR, T cell receptor; MHC II, Class II Major Histocompatibility Complex.

2.2.1 Activation of mature B cells

Based on the primary anatomic location and the requirement of T cell help for activation, in mammals three major subsets of mature B cells: B-1, marginal zone (MZ), and follicular (FO) B cells are distinguished (Allman and Pillai, 2008; Carsetti et al., 2004) (Table 2). The B-1 cells and MZ B cells are fast responders to T-independent antigens (Martin et al., 2001) met in peritoneal and pleural cavities, at mucosal sites, and marginal sinuses of the spleen. Their antigen receptors have limited diversity and give rise to low-affinity IgM antibodies.

Table 2. Characteristics of main subsets of mature B cells

Subset name	Anatomic location	Renewal	Activating antigens	Activation	BCR editing	Response time	Antibody affinity
B-1 cell	Peritoneal and pleural cavities, at mucosal sites	Self-renewing	Polysaccharides, lipids, nonprotein antigens	TI[a]	Limited SHM[b] and CSR[c]	Hours to few days	Low-affinity IgM or IgG3
MZ[d] B cell	Marginal sinus of the spleen	Continuously renewed from bone marrow	Blood-borne pathogens (bacterial polysaccharides, lipids)	TI[a]	Limited SHM[b] and CSR[c]	Hours to few days	Low-affinity IgM or IgG3
FO[e] B cell	Lymphoid follicles of spleen and lymph nodes	Continuously renewed from bone marrow	Proteins, peptides and nonprotein antigens	Predominantly TD[f]	SHM and CSR	Days to many weeks	High-affinity Ab[g] of different classes

[a)] TI, T-cell-independent; [b)] SHM, somatic hypermutation; [c)] CSR, class-switch recombination; [d)] MZ, marginal zone; [e)] FO, follicular; [f)] TD, T-cell-dependent; [g)] Ab, antibody

The B-1a cells (a subclass of B-1 cells) continuously produce polyreactive natural (pre-existing) antibodies critical for innate immune responses constituting the first line of defense (Avrameas, 1991; Ochsenbein et al., 1999). During an active immune response, the MZ B cells can deliver antigens to the junction of the T-cell-rich zone and B-cell follicles of the spleen (Cinamon et al., 2008). The FO B cells are the predominant group of mature B cells in the lymphoid follicles of spleen and lymph nodes. From peripheral tissues soluble antigens (smaller than 70 kDa) are quickly (within 2 hours) delivered with lymphatic fluid or blood into lymphoid follicles (Batista and Harwood, 2009). Large antigens (such as bacteria, viruses and microorganisms) are retained in the subcapsular sinus of the lymph node by follicular dendritic cells and macrophages (so-called antigen presenting cells) that capture and present antigens to B cells by tethering through complement (CD35 and CD21) or Fcγ receptors (Carrasco and Batista, 2007; Junt et al., 2007; Phan et al., 2007). FO B cells are continuously renewed from bone marrow (Forster and Rajewsky, 1990) and are specialized in response to protein antigens requiring T cell help ($CD4^+$ T cell). The protein antigen binding to BCR is endocytosed, proteolyzed in endosomal vesicles, and displayed in the form of peptides on Class II Major Histocompatibility Complex (Class II MHC) molecule ready to present it to $CD4^+$ T cell (Figure 4).

Upon antigen internalization, FO B cells migrate to the border of the follicle and T cell rich compartment searching for co-stimulatory signals from $CD4^+$ T cells required for activation. This migration is driven by chemotaxis. Stromal T cells secrete CCL19 and CCL21 that attract FO B cells bearing CC chemokine receptor 7 (CCR7) (Reif et al., 2002). After initial priming by dendritic cells $CD4^+$ T cells upregulate expression of CD40 ligand (CD40L), reduce expression of CCR7 and start robust expression of CXC-chemokine receptor 5 (CXCR5) attracting these T cells toward B cell follicles rich in chemokine CXCL13 produced by follicular dendritic cells (FDC) (Allen and Cyster, 2008). These cooperated opposite changes in expression of chemokine receptors allow a B cell that carries an antigen to meet activated T cell at the edges of lymphoid follicles.

The recognition of antigen presented on the B-cell surface MHC II by $CD4^+$ T cell and ligation of CD40L with CD40 (constitutively expressed on B cells), and co-stimulatory molecules: CD28 with CD86, inducible T-cell co-stimulator (ICOS) with ICOS ligand (ICOSL), and programmed cell death protein 1 (PD1) with PD1 ligand (PD1L) stimulates B-cell survival, proliferation, and differentiation [reviewed in (Crotty, 2015; Tangye et al., 2013)] (Figure 4). The T_{FH} cell-induced proliferation of B cells occurs in the outer part of B cell follicle (Coffey et al., 2009). Some of these B cells will give rise to the short-lived plasmablast, and unswitched memory B cells (Taylor et al., 2012), and some will coalesce into compact clusters in the center of the B cell follicle to result in the

GC (Figure 3). The exact signals that determine the direction of the differentiation of antigen-activated B cells remain so far unknown.

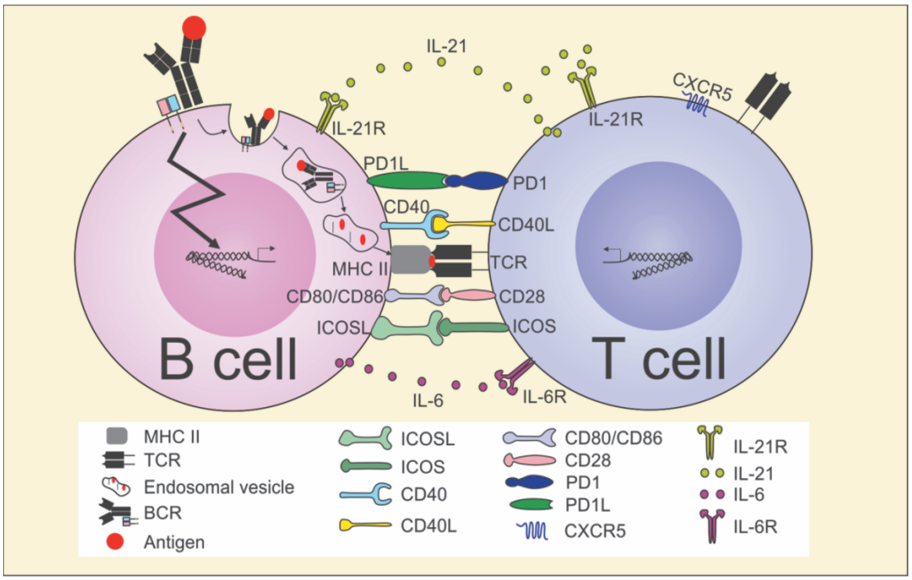

Figure 4. Schematic presentation of B cell—T cell activation in the interfollicular, or T cell zone of the spleen, or lymph node

Upon interaction with antigen presenting dendritic cells, CD4+ T cells acquire the expression of B cell lymphoma 6 (BCL6), CD28, and CXC-chemokine receptor 5 (CXCR5) to differentiate into early T follicular helper (T_{FH}) cells. These T_{FH} cells migrate to the border of the follicle (due to CXCR5 expression) to interact with FO B cells displaying processed antigen on class II Major Histocompatibility Complex (MHC II). The figure schematically presents internalization of the BCR-bearing antigen, BCR-antigen complex processing in the endosomal vesicle and presentation on MHC II. T cell receptor (TCR)-mediated recognition of peptide antigen displayed on MHC II leads to the expression of CD40 ligand (CD40L) and secretion of interleukin 21 (IL-21) by T_{FH} cell. Engagement of CD40 (expressed by B cell) with CD40L (expressed by T cell) stimulates B cell survival, proliferation (clonal expansion) and differentiation. CD40L-mediated signals in combination with different cytokines secreted by T_{FH} cells (IL-21, IL-10, IL-4) enhance B cell proliferation and also can induce CSR. In turn, the secretion of IL-6 by B cells promote the production of IL-21 by T cell that with extended signaling via TCR upregulates expression of programmed cell death protein 1 (PD1), CXCR5 and inducible T-cell co-stimulator (ICOS). ICOS-ICOS-ligand (ICOSL), CD28-CD86 and PD1-PD1 ligand (PD1L) interactions are necessary for the optimal activation and differentiation of both B and T_{FH} cells. [Reviewed in (Tangye et al., 2013)].

2.2.2 BCR signaling in B cell activation

Mature primary B cells have over 300 000 BCR molecules on their surfaces of which 20 000- 150 000 are IgM and 250 000- 300 000 are IgD (Mattila et al.,

2013). BCR molecules densely cover the B cell surface in the form of nanoclusters (20-120 BCR molecules), already in a resting state (Mattila et al., 2013). The exact function of nanocluster structures in a resting state of B cell is under debate and possibly may permit tonic BCR signaling required for survival (Kuokkanen et al., 2015; Mattila et al., 2013).

After recognition of antigen, the immunoreceptor tyrosine-based activating motifs (ITAMs) of the Igα and Igβ subunits of the BCR are phosphorylated by the Src-family tyrosine kinase Lyn within seconds. Phosphorylated ITAMs recruit SYK, which in turn activates several other signaling molecules such as BLNK, VAV, BTK, PLCγ2 and BCAP (Castello et al., 2013; Sohn et al., 2008; Weber et al., 2008). This signalosome leads to the activation of downstream signaling events, including Ca^{2+} release from endoplasmic reticulum (so-called Ca^{2+} flux), and activation of AKT, ERK, MAPK and NF-κB pathways triggering transcriptional changes [reviewed in (Baba and Kurosaki, 2011; Dal Porto et al., 2004; Kurosaki et al., 2010)] (I, Figure S1).

The signaling molecules allow coordinated assembly and development of the dynamic BCR-antigen microclusters initiating the immunological synapse formation (Batista et al., 2001). The early phase of the immunological synapse formation is characterized by a spreading response of B cells that is observed as an actin depolymerization and rapid diffusion of BCRs. Probably B cells try to capture more antigens. The maturation of immunological synapse is followed by B cell contraction response caused by actin polymerization leading to clustering of antigen-bearing BCRs (Fleire et al., 2006). The disruption of actin cytoskeleton induces BCR signaling even in the absence of an antigen and enhances diffusion of the BCRs on B cell surface (Treanor et al., 2010), demonstrating the crucial role of actin in mediating antigen capture and maintenance of BCR signaling threshold. Further experimental work is required to understand the molecular mechanism underlying cytoskeletal changes during B cell activation. What are the factors regulating this process? Are transcription factors involved? It is known that encountering of antigen by BCR initiates a cascade of signaling events that induces drastic changes in gene expression needed for B cell differentiation.

The higher the concentration or the antigen affinity, the more antigens, and BCRs are collected in the microclusters (Liu et al., 2010) and stronger mechanical forces used for physical extraction of antigen from the presenting cells (Natkanski et al., 2013). The internalization of BCR-antigen complex is accompanied by continues BCR signaling. Phosphorylated Syk and Lyn stay with BCR-antigen complex until its trafficking into lysosomes, and while inside, the different kinases are activated (such as p-38, JNK, and ERK) (Chatterjee et

al., 2012; Chaturvedi et al., 2011). In the lysosomes, the antigen is digested into peptides which then are loaded onto MHC II and transported to the surface to display them to $CD4^+$ T cells (Figure 4). The more antigen extracted, the higher densities of peptide-MHC II are presented to $CD4^+$ T cells (Victora et al., 2010). And results in stronger help via co-stimulatory signals from $CD4^+$ T cells for B cell activation (discussed earlier) and differentiation (discussed in the following chapter).

2.2.3 Germinal center

Germinal center (GC) is a microanatomic structure emerging within secondary lymphoid organs upon antigen exposure and is important for affinity maturation and CSR of antigen-activated B cells (Berek et al., 1991). The B cell affinity maturation is achieved by rounds of SHMs followed by a selection of highest affinity BCRs toward antigen which in GC is presented by follicular dendritic cells (FDC). The selection is achieved by competition between B cells in obtaining T cell help critical for B cell survival. B cells that fail to obtain either antigen-mediated immunoglobulin signal or help from T_{FH} cell undergo apoptosis in the GC (Liu et al., 1989). The affinity and amount of produced antibodies against protein antigen increases during an immune response. As a result, the amount of antigen decreases. The surviving B cells need to challenge decreasing amount of antigen leading to a constant increase in BCR affinity. Thus, the affinity of antibodies increases until the infection is eradicated. The feature of GC rapid proliferation coupled with active genomic mutations poses a risk of developing different types of B-cell lymphomas [reviewed in (Basso and Dalla-Favera, 2015)].

The fraction of vigorously proliferating antigen-activated B cells (T cell-mediated activation) that is meant to undergo GC reaction moves to the B cell follicle by changing B-cells migratory properties (Gatto et al., 2009; Pereira et al., 2009). Intensively dividing B cells within a network of FDCs in the center of the follicle give rise to the early GC. FDCs provide long-term storage of entire antigen within complement-coated immune complexes that allow BCR affinity testing (Heesters et al., 2014). Furthermore, activated through Toll-like receptor 4 (TLR4) FDCs support survival of GC B cells and generation of class-switched high-affinity plasma cells and memory B cells. In the absence of TLR4 function, FDCs cannot mature and upon immunization such as mice produce smaller GCs and lower titers of class-switched antigen-specific antibodies than control animals (Garin et al., 2010).

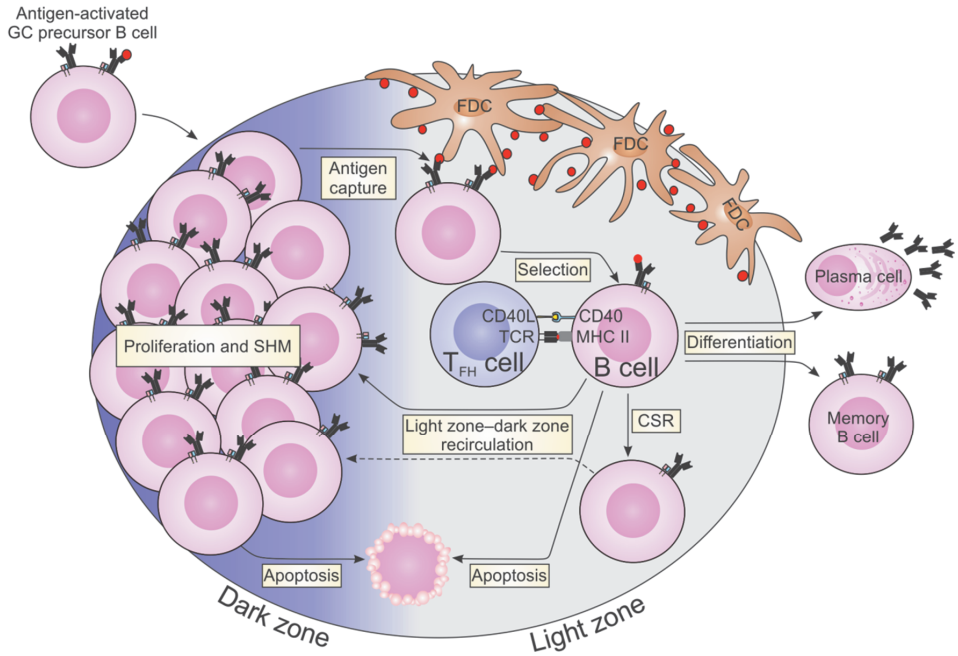

Figure 5. Schematic presentation of germinal center reaction
Antigen-activated germinal center (GC) precursor B cell intensively proliferates forming GC. In the dark zone of GC B cells continue extensive proliferation coupled with the introduction of point mutations into variable region of rearranged heavy and light chain of immunoglobulin genes by somatic hypermutation (SHM). B cells with mutated B cell antigen receptor (BCR) migrate into the light zone of GC to test it for antigen affinity. Upon antigen capture, B cell presents processed antigen on Class II Major Histocompatibility Complex (MHC II) to follicular helper T cell (T_{FH}) and depending on obtained help can differentiate into a plasma cell, memory B cell or undergo additional rounds of proliferation and SHM. The interleukins secreted by T_{FH} cells can induce class-switch recombination (CSR), and such B cell can differentiate into plasma cell or memory B cell, or re-enter dark zone. B cells that cannot retrieve an antigen from follicular dendritic cells (FDC), or become autoreactive, or cannot obtain help from T_{FH} cell undergo apoptosis. TCR, T cell receptor.

Mature GCs have two distinct zones based on the traditional DNA staining in histology: dark zone (DZ) and light zone (LZ) (Figure 5). The DZ is compact, enriched in highly proliferative B cells called centroblast and thus darker due to the higher DNA content than the LZ. In the LZ, B cells called as centrocytes are sparsely distributed among FDCs, T_{FH} cells, and macrophages (Allen et al., 2007a). DZ B cells are characterized by high expression of chemokine receptor CXCR4 (Allen et al., 2004; Allen et al., 2007b; Victora et al., 2012; Victora et al., 2010) which attract these cells to the CXCL12-expressing reticular cells in the DZ, away from FDCs (Bannard et al., 2013; Rodda et al., 2015). The gene expression profile of centroblasts shows a high expression of genes involved in SHM of immunoglobulin variable regions such as *AICDA*, *POLH*, *E2A* and *BCL6* (McHeyzer-

Williams et al., 2015; Victora et al., 2012; Victora et al., 2010). The role of *AICDA*, *E2A*, and *BCL6* in SHM and GCV is shown in Table 1. *POLH* encodes an error-prone DNA polymerase eta that generates A: T mutations during SHM (Masuda et al., 2008). The LZ B cells upregulate the expression of CXCR5 and genes associated with activation phenotypes such as activation markers *CD86* and *CD83*, as well as genes induced by CD40 and BCR pathways and MYC (Allen et al., 2004; Dominguez-Sola et al., 2012; Victora et al., 2012; Victora et al., 2010).

The DZ and LZ separate proliferation and SHM from antigen-driven selection and CSR, respectively in a spatiotemporal context (Figure 5). GC B cells with mutated BCR migrate from the DZ to the LZ to test it for the affinity to an antigen displayed by FDC in a highly competitive manner. Those B cells which manage to retrieve an antigen in BCR affinity-driven manner from FDC, present it to T_{FH} cell (Batista and Neuberger, 2000). The limited number of T_{FH} cells drives the GC selection, as GC B cells compete via presentation of different densities of peptide-MHC II for T cell help and survival signals (Allen et al., 2007b; Batista and Neuberger, 2000; Gitlin et al., 2015; Gitlin et al., 2014; Meyer-Hermann et al., 2006; Radmacher et al., 1998; Shulman et al., 2013; Victora et al., 2010). In the absence of survival signals provided for example by CD40-CD40L ligation or attachment to FDC, GC B cells undergo apoptosis (Elgueta et al., 2009; Koopman et al., 1997; Kosco et al., 1992; Lindhout et al., 1993).

Already more than 10 years ago, it was predicted that gradual increase of BCR affinity requires cyclic re-entry of selected GC B cells from LZ to DZ (Kepler and Perelson, 1993; Oprea and Perelson, 1997). With modern imaging techniques, it was experimentally possible to prove (Allen et al., 2007b; Gitlin et al., 2014; Schwickert et al., 2007; Victora et al., 2010). 10-30% of B cells that enter LZ returns to the DZ and the rest of them exit the GC or die (Meyer-Hermann et al., 2012; Victora et al., 2010). In addition to the regulation of cyclic re-entry, T cell help determines the cell cycle speed and the number of divisions of selected B cell. The interaction between B cell and T_{FH} cell mediated by surface molecules (Figure 4) not only protects B cell from apoptosis but also dictate the number of cell divisions which B cell will undergo in the DZ. The stronger B cell: T cell interaction is in the LZ, the more cell divisions B cell can undergo in the DZ without returning to the LZ (Gitlin et al., 2015; Gitlin et al., 2014). Thus T_{FH} cell help seems to set the “counter” or “timer” (Bannard et al., 2013) for the residency of positively selected GC B cell in the DZ.

The GC B cell: T cell interaction was also found to create a positive feedback loop. The ICOSL on GC B cell upregulates the expression of CD40L on T_{FH} cell, and CD40L upregulates ICOSL (Liu et al., 2015). Entanglement of receptors with ligands promotes B-T cell connection on a large surface area and can en-

hance the T cell production of cytokines IL-21, IL-4, and BAFF. The latter was found to endorse survival of B cells that obtained mutations increasing the affinity of BCR (Goenka et al., 2014). Cytokines secreted by T_{FH} cells not only stimulate the B cell proliferation but can also induce AID expression and CSR (Dedeoglu et al., 2004; Zhou et al., 2003). CSR changes an antibody effector function by excision of intervening IgH sequence between switch regions, thus switching to another Ig class such as IgG, IgE or IgA (Figure 2).

The role of BCR signaling in GC is not clear, as most of the GC B cells are unresponsive to the soluble antigens. Only a brief phosphorylation of Syk is observed in DZ (Khalil et al., 2012). Furthermore, acute strong induction of BCR triggers apoptosis in GC B cells (Han et al., 1995; Pulendran et al., 1995; Shokat and Goodnow, 1995). Immobilization of antigen in experimental settings activates signaling downstream of the BCR but without nuclear translocation of NF-κB p50 subunit for which CD40 ligation is required (Nowosad et al., 2016). *In vivo* studies with application of sensitive reporter show that small fraction of LZ B cells activates BCR engagement, and these cells express *MYC*, *CCND2*, and *IRF4* (Mueller et al., 2015).

2.2.4 Post-germinal center differentiation

The antibody-secreting plasma cells and memory B cells are the developmental outcome of post-germinal center differentiation. This outcome depends on GC B cell affinity toward antigen and T cell help. The higher affinity facilitates plasma cell differentiation both at the T: B border and within GCs (Paus et al., 2006; Phan et al., 2006; Smith et al., 1997). However, GC B cells with a lower-affinity may preferentially differentiate into memory B cells (Shinnakasu et al., 2016). The affinity-driven differentiation similarly to the selection and cyclic re-entry between LZ and DZ may depend on the signals from BCR and T_{FH} cell. Studies with lectin DEC-205 antigen targeting (T cell antigen loaded on the GC B cell's MHC II by fusion with antibody to DEC-205) evidenced that increased T cell help alone can trigger plasma cell differentiation in this experimental set up (Schwickert et al., 2011; Victora et al., 2010). The tendency of B cells to differentiate into plasma cells or memory B cells changes during the antibody response. Memory B cells arise mostly from the pre-GC and early GC B cells whereas long-lived plasma cells originate from late GC response (Weisel et al., 2016). This is in agreement with the increase of affinity during GC reaction and since memory B cells need to stay polyreactive to be able to recognize not only the same pathogen but also different variants upon re-infection. The exact mechanism of affinity-driven differentiation is poorly understood and whether

the differentiation occurs in LZ just after obtaining signals from T_{FH} cell, before the proliferation or in DZ upon few rounds of proliferation is also unclear.

Based on cell migration dynamics the plasma cells may origin from DZ (Meyer-Hermann et al., 2012) but a number of studies propose that plasma cell differentiation starts in LZ where a small subset of B cells expressing IRF4 and BLIMP1 (a master regulator of plasma cell fate) is found (Angelin-Duclos et al., 2000; Falini et al., 2000). An asymmetric inheritance (during cell division) of endocytosed antigen (Thaunat et al., 2012) or smaller concentration of key GC molecules such as IL-21 receptor and BCL6 (Barnett et al., 2012) may also contribute to the choice of post-GC fate. Furthermore, the signaling through CD40, BCR, and TLR can activate NF-κB leading to IRF4-mediated repression of *BCL6* (a master regulator of GC) (Saito et al., 2007). BCR-induced MAPK increases BCL6 phosphorylation and subsequent degradation (Niu et al., 1998). Thus, via elimination of BCL6, the balance is tipped toward plasma cell differentiation and plasmacytic transcriptional program characterized by a high expression of IRF4 and BLIMP1.

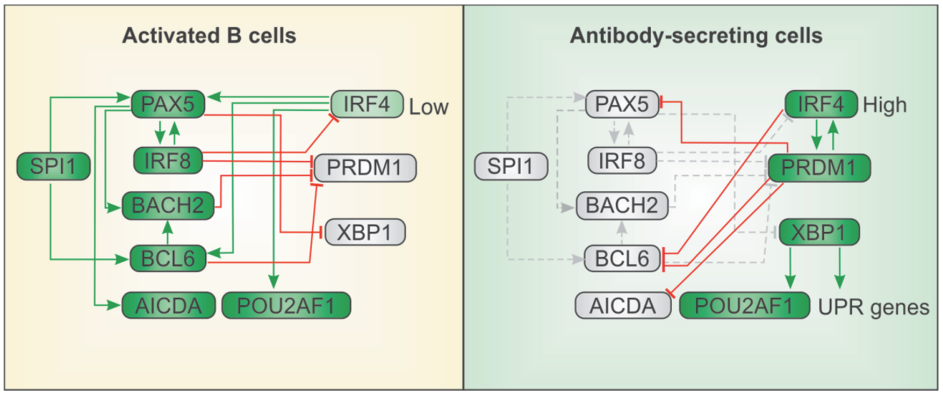

Figure 6. Model of the gene regulatory network in activated B cells and antibody-secreting cells (plasmablasts and plasma cells) controlling B cell terminal differentiation
Expressed genes are shaded green, and genes not expressed or expressed at low levels are shaded gray. The green lines with arrows, red lines, and gray dotted lines indicate direct activation, repression or regulatory mechanism downregulated during terminal differentiation into antibody-secreting cells, respectively. SPI1, Spleen Focus Forming Virus (SFFV) Proviral Integration Oncogene (encoding PU.1); PAX5, Paired box protein 5; IRF8, Interferon Regulatory Factor 8; BACH2, BTB and CNC homologue 2; BCL6, B cell lymphoma 6; AICDA, Activation-induced cytidine deaminase (encoding AID); POU2AF1, POU Class 2 Associating Factor 1 (encoding OBF1); IRF4, Interferon Regulatory Factor 4; BLIMP1, B lymphocyte-induced maturation protein 1 (encoded by PRDM1); XBP1, X-box-binding protein 1; UPR, unfolded protein response. Direct repression of IRF4 by IRF8 (Xu et al., 2015) and AICDA by BLIMP1 (Minnich et al., 2016) have been recently shown and other interactions are reviewed in (Alinikula and Lassila, 2011; Nera et al., 2015; Nutt et al., 2015).

The transition from GC state to antibody-secreting plasma cell requires coordinated adjustments in the expression of thousands of genes. This includes the loss of B cell gene expression (*PAX5*, *BCL6*, and *BACH2*) and gain of expression of genes activating antibody-secretion (*IRF4*, *PRDM1*, and *XBP1*) (Nera et al., 2015; Nutt et al., 2015; Shi et al., 2015). Mutually antagonistic interactions occur between these distinct transcriptional programs: the B cell genes repress antibody-secreting program and factors of secreting program repress the B cell genes (Figure 6). Although the major transcriptional program is known, the contribution of external signals (from the antigen, $CD4^+$ T cells, cytokines) into modulation of terminal B cell differentiation is poorly understood.

2.3 Transcription factors in B cell development

The development of hematopoietic stem cells toward mature B cells and terminally differentiated plasma cells is a complex process tightly regulated by transcription factors (Figure 7). The gene targeting studies in mice demonstrate the importance of transcription factors for B-cell development and function.

The disruption of either Ikaros (Wang et al., 1996), PU.1 (Scott et al., 1994), E2A (Bain et al., 1994), EBF (Lin and Grosschedl, 1995) or PAX5 (Urbanek et al., 1994) results in a developmental blockage at early stages of B-cell differentiation showing their importance for B cell commitment and maintenance of B cell phenotype. Interestingly, deletion of *PAX5* in DT40 cells leads to plasma cell differentiation (Nera et al., 2006b). The disruption of either *Bcl6* (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997), *Bach2* (Muto et al., 2004) or *Irf4* (Mittrucker et al., 1997; Ochiai et al., 2013; Willis et al., 2014) impairs the formation of germinal centers. Furthermore, deletion of *Irf4* (Mittrucker et al., 1997) or *Prdm1* (Shapiro-Shelef et al., 2003) prevents B cells from terminal differentiation into antibody-secreting plasma cells.

IRF4 is expressed in almost all B-cell developmental stages except centroblasts (Cattoretti et al., 2006; Falini et al., 2000; Willis et al., 2014). The IRF4 expression in centrocytes is unclear. The initial studies by Falini et al. reported IRF4 protein expression in a small subset of GC B cells (Falini et al., 2000), while in subsequent studies *Irf4* mRNA was barely detectable in the RNA sequencing of the GC B cells (Willis et al., 2014). This discrepancy may result from different methods used for IRF4 detection. The *Irf4* mRNA from few positive cells could be undetectable due to “dilution-like” effect in the whole GC population of B cells.

IRF4 expression pattern is reciprocal to IRF8. IRF4 is weakly expressed in naïve B cells, induced to an intermediate levels in activated B cells (with the exception

of B cells differentiating into plasmablasts) and together with BLIMP1 (encoded by *PRDM1*) and XBP1 is very highly expressed in antibody-secreting plasma cells (Carotta et al., 2014; Ochiai et al., 2013; Sciammas et al., 2006; Willis et al., 2014). In contrast, other B cell phenotype maintaining factors such as IRF8, BACH2, and PAX5 are no longer expressed in plasma cells (Figure 7).

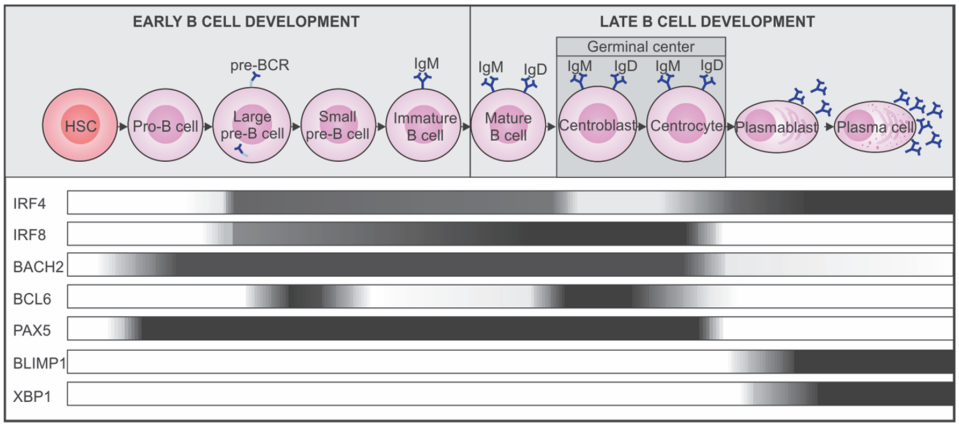

Figure 7. The expression levels of the major transcription factors during B cell differentiation

The color intensity responds to expression level, where black indicate high expression and white no expression. HSC, hematopoietic stem cell.

The expression pattern of BACH2 is like that of PAX5; both factors are expressed from multipotent progenitor to mature B cell stage (including centroblast and centrocyte) and absent in plasma cells (Figure 7). PAX5, FOXO1, and E2A activate the *BACH2* expression at the transition from multipotent progenitor to pro-B cell stage (Casolari et al., 2013; Lin et al., 2010; McManus et al., 2011). However, *BACH2* expression can often precede that of *PAX5* and *EBF1* (Itoh-Nakadai et al., 2014).

BCL6 is a transcription factor with a limited expression to pre-B cells and GC B cells (Figure 7). The expression of functional pre-BCR induces *BCL6* expression which continues in small pre-B cells to protect the cells undergoing Ig light chain rearrangement from DNA damage-induced apoptosis (Duy et al., 2010; Nahar et al., 2011). The low mRNA level of *BCL6* is observed in resting mature B cells, moderately increased in germinal center B cells, and absent in plasma cells (Allman et al., 1996; Cattoretti et al., 1995). However, the highest expression of BCL6 protein occurs in GC and more specifically in highly proliferative centroblasts (Kuo et al., 2007). The expression pattern suggests a significant role for BCL6 in these cells. Indeed, GCs are not formed in the absence of *Bcl6* (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997).

2.3.1 IRF4

Interferon Regulatory Factor 4 (IRF4) (also known as Pip, MUM1, NF-EM5, LSIRF and SHEP8) belongs to the interferon regulatory factor (IRF) family of transcription factors. It has a conserved tryptophan pentad repeat DNA-binding domain (Escalante et al., 2002; Taniguchi et al., 2001) and C-terminal regulatory domain (Figure 8).

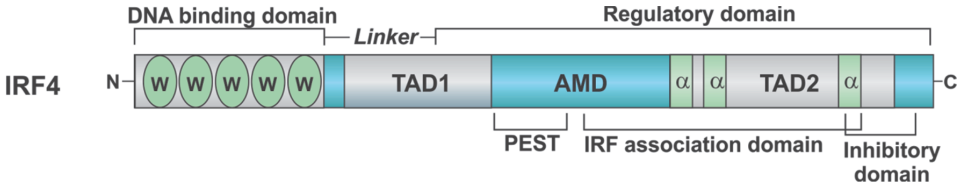

Figure 8. Schematic presentation of the IRF4 protein
The IRF4 protein consists of three main functional domains: DNA binding domain, regulatory domain (composed of two transactivation domains (TAD1-2), activation masking domain (AMD) and inhibitory domain) and a linker domain allowing conformational changes of the IRF4 protein. PEST, peptide sequence rich in proline, glutamic acid, serine, and threonine mediates protein stability and activity. W, tryptophan; α, α-helix;. Modified from (De Silva et al., 2012).

In B cells, T cells, macrophages or dendritic cells, IRF4 binds DNA as a heterodimer complex with a number of cofactors: PU.1, SPIB, E47, IRF8, NFAT, FOXP3, STAT proteins, MYD88, IBP, and BATF (Eisenbeis et al., 1995; Ettinger et al., 2005; Gupta et al., 2001; Gupta et al., 1999; Hu et al., 2002; Kwon et al., 2009; Li et al., 2012b; Meraro et al., 2002; Nagulapalli and Atchison, 1998; Negishi et al., 2005; Pongubala et al., 1992; Rao et al., 1999; Rengarajan et al., 2002; Rosenbauer et al., 1999; Su et al., 1996; Tussiwand et al., 2012; Zheng et al., 2009). The IRF4 interactors can shape its activity either as activator or repressor (De Silva et al., 2012). In the absence of cofactors, IRF4 is unable to bind DNA because its DNA binding domain is masked by IRF association domain (Yamagata et al., 1996) (Figure 8). However, Ochiai et al. demonstrated that IRF4 could bind DNA as a homodimer at high IRF4 concentrations (Ochiai et al., 2013). The selection of dimerizing partner by IRF4 may have an impact on the choice of the target gene and regulatory outcome.

2.3.1.1 The redundant function of IRF4 and IRF8 in early B cell development

Regarding primary structure and cellular expression pattern (Figure 7), IRF4 is most closely related to IRF8 (Interferon Regulatory Factor 8). In IRF4 and IRF8 (*Irf4*$^{-/-}$/*Irf8*$^{-/-}$) double deficient mice B cell development is arrested at large pre-B cell stage (Lu et al., 2003) (Figure 9). These *Irf4*$^{-/-}$/*Irf8*$^{-/-}$ B cells are hyperprolif-

erative and fail to induce expression of the Ikaros family factors (Ikaros and Aiolos) (Ma et al., 2008). The IRF4 expression is induced by pre-BCR signaling in large pre-B cells (Thompson et al., 2007) (Figure 9). The downregulation of the pre-BCR and withdrawal from cell cycle is required for B cell developmental progression from large pre-B cell to small pre-B cell stage. Ikaros and Aiolos are required to mediate this process (Gomez-del Arco et al., 2004; Ma et al., 2010; Sabbattini et al., 2001; Thompson et al., 2007).

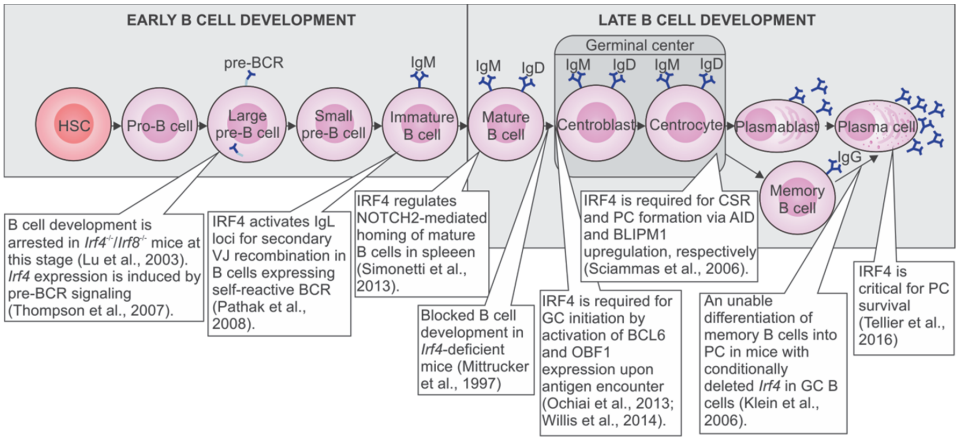

Figure 9. Main functions of IRF4 during murine B cell development
HSC, hematopoietic stem cell; PC, plasma cell; GC, germinal center; BCR, B cell antigen receptor; CSR, class-switch recombination.

IRF4 also indirectly limits the expansion of large pre-B cells by attenuating IL-7 receptor signaling known to repress the light chain recombination (Mandal et al., 2011) in these cells via induction of chemokine CXCR4 expression (Johnson et al., 2008). This chemokine attracts large pre-B cells toward CXCL12 expressing stromal cells that are located away from IL-7 secreting stromal cells which support large pre-B cells proliferation (Tokoyoda et al., 2004). The complementation of the *Irf4*$^{-/-}$/*Irf8*$^{-/-}$ pre-B cells with either *Irf4* or *Irf8* was sufficient to rescue these cells from developmental arrest (Ma et al., 2006) showing that in early B cell development IRF4 and IRF8 function redundantly. However, at the immature B cell stage, the defect in the secondary Ig rearrangements induced by self-antigen can be specifically seen in the *Irf4*- but not in *Irf8*-deficient mice (Pathak et al., 2008). The self-reactive BCR uniquely induces IRF4 which in turn activates light chain loci for secondary Ig gene rearrangements (Pathak et al., 2008) (Figure 9). IRF4 despite redundant function with IRF8 in downregulation of pre-BCR expression has a unique role in the elimination of self-reactive BCR.

2.3.1.2 The role of IRF4 in homing of mature B cells

IRF4 seems to be also involved in the homing properties of mature B cell, but the exact mechanism of its action is unclear. The inducible deletion of *Irf4* specifically in mature B cells revealed that IRF4 regulates NOTCH2-mediated homing of mature B cells either in MZ or FO compartment of the spleen (Simonetti et al., 2013) (Figure 9). This study is in agreement with an initial observation from *Irf4*$^{-/-}$ germline mutant indicating a changed proportion between FO B cells and MZ B cells towards an increase in the MZ subset (Klein et al., 2006; Mittrucker et al., 1997; Ochiai et al., 2013). NOTCH signaling is a prerequisite for the development and the maintenance of MZ B cells (Pillai and Cariappa, 2009). Thus, upregulated NOTCH2 expression and activity in the absence of *Irf4* is most likely responsible for the retention of mature B cells (FO and MZ B cells) in the MZ area. Simonetti et al. showed that indeed inhibition of NOTCH2 with an NOTCH2-inhibiting antibody could release the FO B cells from the MZ area. They also reported the changed expression of chemokine receptors and integrins associated with the trafficking of MZ B cells (Simonetti et al., 2013). However, the exact mechanism how IRF4 regulates NOTCH2 remains unknown.

Another study conducted in heterozygous *Irf4*$^{+/-}$ mice (New Zealand Black mice) characterized by low IRF4 levels reported a hyperproliferative phenotype of B-1 cells with an accumulation of B-1a cells in peritoneal cavities (Ma et al., 2013). These two studies report that IRF4 has a role in the homing of mature B cells, but more research is required to understand it.

2.3.1.3 The function of IRF4 in late B cell development

The *Irf4* knockout mice have severe defects in a late B cell development (Mittrucker et al., 1997) (Figure 9). These mice have a low serum Ig level and upon immunization cannot form GCs and plasma cells, perform CSR, and produce antigen-specific antibodies despite a normal number of mature B cells in the spleen. The same defect in plasma cell differentiation is observed upon conditional deletion of *Irf4* in GC B cells (Klein et al., 2006) and mature B cells (Ochiai et al., 2013; Willis et al., 2014) and in heterozygous *Irf4*$^{+/-}$ mice (Ma et al., 2013). Interestingly, the ectopic expression of AID in *Irf4*-deficient B cells restores the Ig isotype switching (indicating its limiting role for CSR) (Sciammas et al., 2006) whereas the effect of ectopic expression of BLIMP1 in the same cells is still controversial. In one study, BLIMP1 expression in the absence of IRF4 was sufficient to promote antibody secretion (Sciammas et al., 2006) but in another one, no antibody production was observed (Klein et al., 2006). These

conflicting results raise the important question whether IRF4 and BLIMP1 are dispensable for the plasma cell differentiation.

Ochiai et al. and Willis et al. demonstrated by conditional deletion of *Irf4* gene from mature B cells (before antigen stimulation) that IRF4 is critical for the initiation of GC reaction in a B cell-intrinsic manner (Ochiai et al., 2013; Willis et al., 2014) (Figure 9). The short impulse of IRF4 expression for two days (obtained by the use of Tet-inducible *Irf4* transgene) could induce GC formation in *Irf4*$^{-/-}$ B cells as well as the expression of BCL6 and AID but not OBF1 (Ochiai et al., 2013). This short impulse was also insufficient to generate plasma cells, as plasma cells were observed only in mice continuously expressing IRF4. Furthermore, this study showed *in vivo* and *in vitro* that the intensity of BCR signaling triggers the IRF4 expression, as antigens binding with high-affinity to BCR boost greater IRF4 expression leading to the generation of plasma cells (Ochiai et al., 2013; Sciammas et al., 2011). The isotype of the BCR can influence the expression level of IRF4 as well. B cells with BCR of IgE isotype express more IRF4 than those with IgG1 and can differentiate into short-lived plasma cells even without the antigen induction (Haniuda et al., 2016).

The different IRF4-dependent functions in the late B cell development (generation of GC and plasma cell differentiation) support an IRF4 concentration-dependent model primarily introduced by Sciammas et al. (Sciammas et al., 2006) and expanded by Ochiai et al. (Ochiai et al., 2013). According to the model, low level of IRF4 expression regulates generation of GC B cells by upregulating expression of BCL6 and OBF1 (encoded by *POU2AF1*) as well as AID; whereas a high IRF4 expression upregulates BLIMP1 (encoded by PRDM1) and promote plasma cell differentiation with simultaneous repression of genes maintaining the B cell program *PAX5*, *BCL6*, and *IRF8* (Klein et al., 2006; Ochiai et al., 2013; Sciammas et al., 2006). These different modes of IRF4 actions are also reflected on its binding preferences. IRF4 in a low concentration binds to the high-affinity Ets-IRF composite motifs (EICE) together with PU.1 or SPIB, and AP-1-IRF composite motifs (AICE) together with BATF. Through these interactions, IRF4 regulates the expression of genes involved in the B cell activation and the GC response (Ochiai et al., 2013). The high IRF4 concentration changes its binding preference, as a homodimer, to the lower affinity canonical interferon sequence response elements (ISRE) present in the genes associated with plasma cell differentiation (Ochiai et al., 2013). While "IRF4 graded expression model" well explains the function of IRF4 in plasma cell differentiation, the exact role of IRF4 in maintaining the plasma cells phenotype beside upregulation of BLIMP1 remains unknown.

Recently, Tellier et al. took an attempt to reveal the function of IRF4 in the plasma cells by conditional inactivation of IRF4 in plasma cells (Tellier et al., 2016). The deletion of *Irf4* from plasma cells resulted in cell death making further discoveries impossible other than that IRF4 is required for plasma cell survival (Tellier et al., 2016) (Figure 9). Interestingly, similar results were observed when IRF4 was knocked down in multiple myeloma cell line with high expression of IRF4. Again, the elimination of IRF4 caused a dramatic decrease in the cell viability (Shaffer et al., 2008). It appears that there is a positive feedback loop between IRF4 and MYC (an oncogene) where oncogenic MYC activates IRF4, and then IRF4 upregulates MYC expression, thus sustaining the multiple myeloma phenotype (Shaffer et al., 2008). Therefore, IRF4 seems to be important for maintaining cell viability of plasma cells.

2.3.2 BACH2

BTB and CNC homology 2 (BACH2) is a basic leucine zipper (bZip) transcription factor which binds DNA through its bZip domain either as a homodimer or heterodimer. BACH2 preferentially binds DNA at the MAF recognition element (MARE) as a heterodimer with MAFK or other MAF family proteins (Oyake et al., 1996). This heterodimerization occurs through the bZip domain. This domain also has a nuclear localization signal (Hoshino et al., 2000) (Figure 10). BACH2 can also heterodimerize with BATF (bZIP transcription factor ATF-like) (Kuwahara et al., 2016) and bind DNA as a homodimer at the AP-1-like sequences, but only at high concentrations (Oyake et al., 1996). The N-terminal BTB/POZ domain of BACH2 mediates interactions with other proteins (Kobayashi et al., 2000).

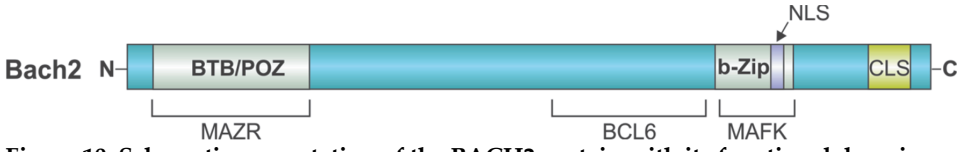

Figure 10. Schematic presentation of the BACH2 protein with its functional domains
BACH2 possesses two domains: BTB/POZ domain mediating oligomer formation (Kobayashi et al., 2000), and bZip domain—a DNA binding domain—involved in dimerization with MAF family factors. Within a bZip domain is situated a nuclear localization signal (NLS) and at C-terminal region of BACH2 is a cytoplasmic localization signal (CLS) (Hoshino et al., 2000). The parts of BACH2 demonstrated to be involved in interaction with: MAZR (MAZ-Related Factor) (Kobayashi et al., 2000), BCL6 (Ochiai et al., 2008) and MAFK (Oyake et al., 1996) are indicated.

2.3.2.1 Post-translational regulation of BACH2

BACH2 undergoes posttranslational modifications. The mouse BACH2 protein has 72 phosphorylation sites. Phosphorylation at single serine (S535) causes BACH2 cytoplasmic accumulation (Ando et al., 2016). In human BACH2, phosphorylation also induces cytoplasmic retention, but via serine 521 which is phosphorylated in response to the PI3K/S6 kinase pathway (Yoshida et al., 2007). The phosphorylation of BACH2 by PI3K-AKT-mTOR pathway seems to be an additional mechanism regulating BACH2 expression. *In vitro* stimulation of B cells induces BACH2 phosphorylation at multiple sites (Ando et al., 2016) and in another study the same stimulation leads to the repression of BACH2 expression (Kometani et al., 2013), linking the BACH2 phosphorylation and suppression. The stimulation-induced repression of BACH2 was lost upon treatment with rapamycin (an inhibitor of mTOR) both *in vitro* and *in vivo* or API-2 (an inhibitor of Akt) *in vitro* (Kometani et al., 2013), suggesting that BACH2 is regulated via an Akt-mTOR pathway. Furthermore, the downstream targets of AKT, FOXO1 seem to be involved in the modulation of BACH2 expression, as a constitutively active form of FOXO1 can reduce the stimulation-induced repression of BACH2 expression (Kometani et al., 2013). The BACH2 protein has 11 phosphorylation sites consensus with mTOR motifs and *in vitro* mTORC1 can directly phosphorylate the recombinant BACH2 (Ando et al., 2016).

The antigen-induced phosphorylation of BACH2 and following nuclear exclusion may directly contribute to the B cell fate decisions. This idea is partly supported by the observations that cells which accumulate BACH2 in the cytoplasmic compartment upon in vitro stimulation undergo plasma cell differentiation without performing CSR (Muto et al., 2010).

2.3.2.2 Intracellular localization of BACH2

Under normal conditions, the majority of BACH2 protein is localized in the cytoplasm. The oxidative stress caused by diethyl maleate (DEM) induces the nuclear retention of BACH2 around promyelocytic leukemia nuclear bodies (nuclear substructures involved in transcriptional regulation during apoptosis and stress responses) (Muto et al., 2002) causing selective repression of transcription activity in this area (Tashiro et al., 2004). The anticancer drug STI571 (an oxidative stressor) induces BACH2 expression (Vieira et al., 2001) and nuclear accumulation of BACH2 associated with the apoptotic cell death and the inhibition of proliferation of chronic myeloid leukemia cells (Kamio et al., 2003). In another study, the overexpression of BACH2 in NIH 3T3 cells inhibits cell proliferation

and causes apoptosis upon oxidative stress in both NIH 3T3 and Raji B lymphoid cell lines (Muto et al., 2002). These studies suggest a proapoptotic role of BACH2 in the oxidative stress responses.

2.3.2.3 The function of BACH2 during early B cell development

In early B cell development BACH2 together with BACH1 promotes the B cell commitment by repression of myeloid genes (Itoh-Nakadai et al., 2014) (Figure 11). Furthermore, BACH2 antagonizes the C/EBP gene regulatory network (a myeloid lineage driver) and activates genes with functions in the lymphoid lineage development (Itoh-Nakadai et al., 2017). After mimicking the infection condition with a lipopolysaccharide (LPS) at multipotent progenitor stage, the BACH2 expression is reduced allowing enhanced myeloid differentiation (Itoh-Nakadai et al., 2017). Nerveless, the function of BACH2 in lymphoid lineage commitment is to modulate/ tune rather than absolutely required because *Bach2* knockout mice do not have a severe defect in early B and T cell development (Muto et al., 2004).

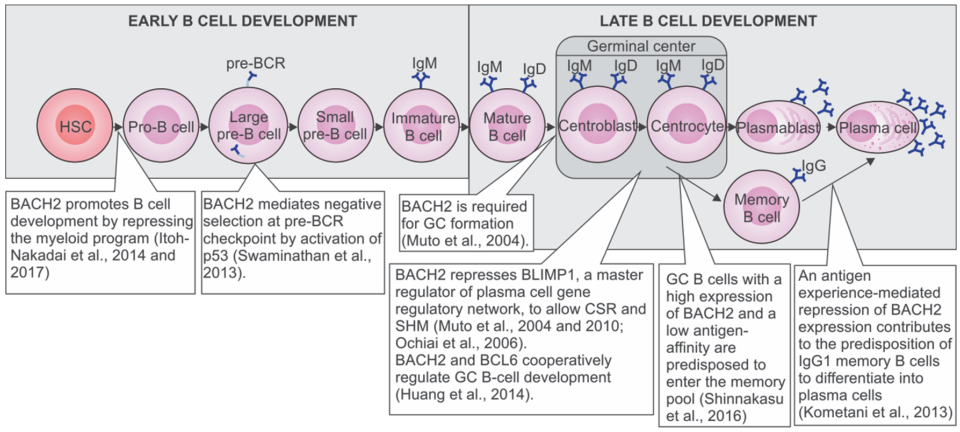

Figure 11. Main functions of BACH2 during murine B cell development
HSC, hematopoietic stem cell; PC, plasma cell; GC, germinal center; BCR, B cell antigen receptor; CSR, class-switch recombination; SHM, somatic hypermutation.

At the pre-B cell stage, BACH2 supports Ig heavy chain V(D)J rearrangements by the direct regulation of *RAG1/2* gene transcription. BACH2 binds to the promoter of *RAG1* and *RAG2* (Swaminathan et al., 2013) and *BACH2* deficiency reduces the mRNA levels of *RAG1/2* leading to lower V(D)J recombination activity. The lack of BACH2 increases the survival of pre-B cells carrying non-functional pre-BCR and re-expression of BACH2 restores this defect (Swaminathan et al., 2013). Swaminathan et al. also found that BACH2 drives the elimination of B cells with non-functional pre-BCR by direct upregulation of

expression of cell cycle checkpoint and DNA damage response genes: *GADD45A*, *GADD45B*, *TP53* (encodes p53) and *CDKN2A* (encodes ARF) that can induce apoptosis, cell cycle arrest, or DNA repair (Figure 11). Interestingly, signaling from successfully formed pre-BCR induces expression of BCL6 which in turn suppresses p53 and ARF expression allowing the Ig light chain rearrangements (Duy et al., 2010).

The chromatin immunoprecipitation followed by sequencing (ChIP-seq) analysis has revealed that at the pre-B cell stage BCL6 and BACH2 share approximately 24% of their target genes (Swaminathan et al., 2013). BACH2 and BCL6 play opposite roles at the pre-B cell stage to maintain the equilibrium between negative selection and survival of pre-B cells. BCL6 permits survival of Philadelphia chromosome-positive ALL (Ph+ ALL) cells through repression of p53 after treatment with an inhibitor of the BCR-ABL1 kinase (Duy et al., 2011). The BCR-ABL1 signaling inactivates PAX5 and subsequently BACH2 expression and activity by inhibition of nuclear translocation (Casolari et al., 2013). In turn, the treatment with BCR-ABL1 inhibitor imatinib upregulates BACH2 expression and nuclear localization in Ph+ ALL cells (Casolari et al., 2013; Ono et al., 2007). The expression level of BACH2 in pre-B cells is crucial for the proper development of B cells, as defects in pre-BCR checkpoint control can cause pre-B acute lymphocytic leukemia (pre-B ALL) (Swaminathan et al., 2013). BACH2 slows down the progression of pre-B ALL by activating cellular apoptosis via p53 upregulation. Thus, the loss of BACH2 expression or function caused by promoter hypermethylation, point mutations in its BTB domain, deletion of the entire locus, or inactivation of PAX5 expression can result in BCL6-mediated repression of p53 (Swaminathan et al., 2013). Together, these results show that BACH2 has an important role in mediating negative selection of pre-B cells with unsuccessful Ig heavy chain rearrangement and in preventing leukemic transformations.

2.3.2.4 The role of BACH2 in late B cell development

Although BACH2 and BCL6 have opposite roles in the pre-B cells, both are critical for the germinal center formation upon antigen activation (Dent et al., 1997; Fukuda et al., 1997; Muto et al., 2004; Ye et al., 1997) (Figure 11). Moreover, the expression of both BCL6 and BACH2 positively correlate in human GC B cells (Huang et al., 2014). Both factors can physically interact in 18-81 pre-B cell line, HEK293 cell line while exogenously overexpressed, in primary human GC B cell line, and in human OCI-LY7 cell line which is a GC-derived diffuse large B-cell lymphoma cell line (Huang et al., 2014; Ochiai et al., 2008) (Figure 10).

Furthermore, BCL6 stabilizes BACH2 protein and cooperates in the transcriptional regulation of GC B cells to sustain GCs and prevent premature differentiation of B cells into plasma cells (Huang et al., 2014). Even that BCL6 and BACH2 bind to distinct consensus motifs on the DNA, the 30% of BACH2 binding sites are shared with BCL6 in OCI-LY7 B cells (screened by ChIP-seq) including binding to the promoters of *PRDM1*, *CD69*, *GADD45A/B*, *MDM2* and *CDKN1B* (Huang et al., 2014). Multiple studies showed that both BCL6 and BACH2 represses *PRDM1* (Alinikula et al., 2011; Huang et al., 2014; Muto et al., 2010; Muto et al., 2004; Ochiai et al., 2006; Shaffer et al., 2002; Shaffer et al., 2000; Tunyaplin et al., 2004; Vasanwala et al., 2002). BCL6 represses *CD69* expression (Shaffer et al., 2000). However, the role of BCL6 and BACH2 binding to other co-occupied genes is not known. Further studies are required to reveal whether they mediate activation or repression of these genes and whether BACH2 and BCL6 act cooperatively or antagonistically like it takes place in pre-B cells. Moreover, it is not known whether BACH2-BCL6 balance protects from the escape of badly harmed—by AID off targeting—GC B cells.

BACH2 mediates the repression of *PRDM1* by a direct binding to the MARE motif at the promoter and intron 5 regions together with MAF proteins (Ochiai et al., 2006). Although ChIP-seq analysis in OCI-LY7 B cell line demonstrate that 52% of BACH2 binding sites are shared with MAFK, neither the *MafK* knock-down in OCI-LY1 cells nor the knockout in mouse affected *PRDM1* expression and GC phenotype (Huang et al., 2014). These findings suggest that other MAF proteins may compensate the loss of MAFK, as BACH2 can interact with other bZip family factors through its bZip domain.

BACH2 allows CSR and SHM by postponing *PRDM1* expression. *Bach2* knock-out mice have the reduced number of mature B cells both MZ and FO B cells and drastically increased levels of their IgM serum was found but not in other Ig isotypes (Muto et al., 2004). Upon LPS stimulation, *Bach2*-deficient murine B cells rapidly differentiate into antibody-secreting plasma cells without performing CSR. These cells have upregulated *Prdm1* and reduced *Aicda* (encoding AID) expressions together with diminished SHM and CSR (Muto et al., 2004). Retroviral complementation of AID in *Bach2*$^{-/-}$ B cells does not restore CSR defect, suggesting that reduced AID level is not the only limiting factor for CSR in the absence of BACH2. A subsequent study showed that the defect in CSR was entirely caused by upregulated expression of *Prdm1*, as deletion of *Prdm1* in *Bach2*-deficient mice restored the CSR (Muto et al., 2010). Therefore Muto et al. proposed a “delay-driven diversity model” in which BACH2 delays the expression of *Prdm1* (and plasma cell differentiation) to allow AID to initiate the SHM/CSR and thus the production of high-affinity antibodies (Muto et al., 2010).

The expression level of BACH2 in late B cell development seems to have an important role in B cell fate decisions. GC B cells with BCR of low affinity and with high BACH2 expression are more predisposed to enter the memory pool than GC B cells of higher antigen affinity and lower *BACH2* level (Shinnakasu et al., 2016). Furthermore, reduced expression of *BACH2* by antigen exposure in IgG1 memory B cells facilitates differentiation into plasma cells (Kometani et al., 2013). Hence the formation and differentiation of memory B cells may depend on BACH2 expression level (Igarashi et al., 2017; Shinnakasu and Kurosaki, 2017).

3 AIMS OF THE STUDY

1. To investigate the role of IRF4 in B cells by using IRF4 deficient DT40 cell line
2. To elucidate the role of IRF4 in antibody secretion
3. To study the role of BACH2 in diversification of immunoglobulin loci by gene conversion and somatic hypermutation

4 MATERIALS AND METHODS

Materials and methods from the manuscripts (I, II, III) are gathered together below and some of them are described in more details.

4.1 Cell culture (I, II, III)

Wild-type (WT), Clone 18 and other mutants of chicken DT40 B cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (Hyclone), 1% chicken serum (BioWest), 50 μM β-mercaptoethanol, 1x GlutaMaxTM-I (Gibco) and 1x Penicillin-Streptomycin (Gibco) in a humidified atmosphere with 5% CO_2 at +40 °C.

4.2 Generation of mutant DT40 B cell lines (I, II, III)

The DT40 mutant cell lines were generated with targeting constructs possessing homologous arms that surrounded the part of the gene intended for the deletion.

4.2.1 IRF4 gene inactivation in DT40 B cell line (I, II)

The *IRF4* knockout (IRF4KO) was obtained in DT40 cell line by disruption of *IRF4* first coding exons with targeting constructs (I, Figure 1A and B). The 1kb left arm of targeting construct was amplified from WT DT40 genomic DNA by PCR using primers C-LF and C-LR (Table 3). The obtained PCR product was digested with *Not*I and *Bam*HI. The 1.2 kb right arm was amplified with primers C-RF and C-RR and digested with *Eco*RI and *Acc*65I. The alternative 1.8 kb right arm used in IRF4KO2 was amplified with primers C-RF2 and C-RR2 and digested with *BamH*I and *Xho*I. The digested arms were cloned into a pBluescript vector. Bsr and Neo selection cassettes flanked with Lox sequences from pLoxBSR and pLoxNeo vectors (Arakawa et al., 2001) and HisD selection were transferred as *BamH*I fragments to the targeting vectors between homologous arms. Obtained vectors were linearized with *Not*I and transfected into DT40 B cells by electroporation using 710 V and 25 μF. Transfected cells were selected in the presence of 30 μg/ml blasticidin (InvivoGen), 2 mg/ml G418 (Sigma) or 1mg/ml L-Histidinol dihydrochloride (Sigma). The deletion of *IRF4* exon two and three was verified by genomic PCR (I, Figure 1C) with primers IRF4_p1 and IRF4_p2. The loss of IRF4 expression was confirmed by Western blot with anti-IRF4 Ab (Table 5, I, Figure 1D).

4.2.2 Genetic complementation of IRF4 expression (I)

Chicken *IRF4* was amplified from WT DT40 cDNA using primers IRF4-f and IRF4-r (Table 3), which created *Hind*III and *Nhe*I sites in PCR product, respectively. Upon digestion, PCR product was cloned between *Hind*III and *Nhe*I sites of the pExpress vector (Arakawa et al., 2001) and then the Puro selection cassette was inserted into *Xho*I site. The resulting plasmid was sequenced and linearized with *Not*I and transfected into IRF4KO cells by electroporation (710 V and 25 μF). Transfected cells were selected in the presence of 0.5 μg/ml puromycin (Gibco), and IRF4 expression within selected clones was verified by Western blotting with anti-IRF4 Ab (I, Figure 1D).

4.2.3 Generation of IRF4/BCL6 double knockout DT40 B cell line (II)

To obtain double deficient cell line in both *IRF4* and *BCL6* (named as DKO), the *BCL6* encoding alleles were removed with previously described targeting vectors (Alinikula et al., 2011) from an IRF4-deficient cell line (IRF4KO1) (I). Before the targeting of *BCL6* gene, the expression vector Mer-Cre-Mer-Puro (Zhang et al., 1996) was stably transfected into IRF4KO cells to allow p-lox mediated removal of Bsr and Neo selection cassettes. The IRF4KO1 cells were induced with 1.29 mM (Z)-4-Hydroxytamoxifen (Sigma) for three days as described (Arakawa, 2006). After that, the cells were single cell subcloned and a clone with removed Bsr, Neo, and Puro selection cassettes was used for further experiments. The absence of IRF4 expression was also confirmed by Western blot analysis (II, Fig. 1A) and the absence of *BCL6* expression by RT-qPCR (II, Fig. 1B).

4.2.4 PRDM1 and IRF4 lentiviral expression constructs (II)

Schematic presentation of expression constructs is presented on (II, Figure 3A, and Figure 4A). Chicken *PRDM1* was amplified from BCL6KO DT40 cDNA using primers Blimp1_F and Blimp1_linker_R. Between *PRDM1* and *Enhanced Green Fluorescent Protein* (*EGFP)* was created linker sequence GGTAGTG-CAGCTGGGAGTAGCGGT. Chicken IRF4 was amplified from WT DT40 cDNA using primers IRF4_F and IRF4_R. The obtained fragments were cloned into a lentiviral vector with In-Fusion HD Cloning Kit (TaKaRa, Clontech) according to manufacturer's instructions.

4.2.5 Infection of cells by lentiviral constructs (II)

293T cells were co-transfected with the psPAX2 and pMD2.G packaging vectors and B1-GFP (p-lenti-RSV-B1/GFP-IRES-PURO), GFP Mock, IRF4mC (p-lenti-RSV-IRF4-T2AmCherry-IRES-GPT) or mC Mock expression plasmids using X-treme GENE HP DNA Transfection Reagent (Roche) according to the manufacturer's instructions. The viral supernatants were harvested three days after the transfection. $2x10^6$ DT40 cells suspended in 200 µl of RPMI 1640 (Sigma) were infected with 200 µl of viral supernatant in the dark for 30 minutes at room temperature. After two days of the infection, cells were grown in a selective DT40 medium. After at least a week of selection, B1-GFP or IRF4mC positive cells were sorted (FACSAria IIu Cell Sorter, BD) and kept in a selective medium. Cells infected with B1-GFP or GFP Mock were grown in the presence of 0.5 µg/ml puromycin (Gibco) and cells infected with IRF4mC, or mC Mock were grown in the presence of 15 µg/ml mycophenolic acid (Millipore), 250 µg/ml xanthine and 20 µg/ml hypoxanthine (Sigma).

Table 3. Primers used for cloning vectors

Primer	Sequence (5' to 3')
C-LF	GGGCGCGGCCGCCTCCATCATATAAAGAAACT
C-LR	ACTCACCCGGATCCAAGTTCTAGCCACTCTTA
C-RF	AGTGTACAGAATAGTGCCAGAAGGAGCTCAAAAAG
C-RR	CTCATGGGGCACCATGTAGTTGGGTACCTATT
C-RF2	CGGATCCTGATATCCCCTACCAGTGTG
C-RR2	CTCAGGAGGGGCTCGAGCATAAAAGGTTC
IRF4_p1	CTGGTGTGGGAGAATGACGAGAAGAGCATC
IRF4_p2	CTCTTGTTCAAAGCACACCTCAATCTGGTC
LA_F	AACAGCGGCCGCATTTACTGCTTGCAGGTAAATAT
LA_R	CACACTAGTGGTCCAGCTGGCTATGGTTTAACTGA
RA_F	ACCTATCGATTTGTAACCTTACCGTTACACCTGGT
RA_R	TCTAGGTACCAACTTTAGCGCAGACCGCAGTAT
Bach2_p1	ACTGTCCTGGCATTTAGAGAATGAACTGCT
Bach2_p2	CTTGGCAGTACCCAAGGAAAATTCTGACAC
IRF4-f	TATAAGCTTATGAACTTGGAGCCGGGTGA
IRF4-r	TTAGCTAGCGGATCTTATTCTTGAATAGAGGAATGG
Bach2_F	TAAAAGCTTATGTCTGTGGATGAGAAGACTGACTC
Bach2_R	TTTGCTAGCCTAGGTGTAATCTTTCCTAGGCTGTT
Blimp1_F	ATTCACCACAGCTAGATGAAGGCTGCTACACGGTGTAGC
Blimp1_linker_R	CTACTCCCAGCTGCACTACCTCCAGGGTCCATTGGTTCAACTGT
IRF4_F	ATTCACCACAGCTAGATGAACTTGGAGCCGGGTGAG
IRF4_R	TTCTTGAATAGAGGAATGGCGAATAGATCTGTGA

4.2.6 Generation of BACH2 knockout DT40 B cell line (III)

The *BACH2* gene was inactivated by targeting constructs shown in article III (III, Figure 1A), which removed BACH2 DNA-binding domain encoded by exon 7 and coding part of exon 8, yielding *BACH2* knockout (BACH2KO) cell line. The 1.8 kb left arm was amplified from WT DT40 genomic DNA by PCR using pri-

mers LA_F and LA_R (Table 3). The PCR product was digested with *Not*I (New England BioLabs) and *Spe*I (New England BioLabs). The 1.9 kb right arm was amplified with RA_F and RA_R and digested with *Cla*I and *Acc65*I. The digested arms were cloned into a pBluescript vector. The floxed Bsr and Neo selection cassettes from pLoxBsr and pLoxNeo vectors (Arakawa et al., 2001) were transferred as *BamH*I (New England BioLabs) fragments to the targeting vectors between homologous arms in an antisense direction to the *BACH2* gene. Targeting vectors were linearized with *Not*I and transfected into DT40 B cells (Clone 18) by electroporation with 700V and 25 µF. Each allele was targeted consequently with Bsr or Neo vectors. The clones were selected in the presence of 30 µg/ml blasticidin (InvivoGen) or 2 mg/ml G418 (Sigma) for neomycin selection. The deletion of exon 7 and coding part of exon 8 was verified by PCR (III, Figure 1B) with primers Bach2_p1 and Bach2_p2 (Table 3). The loss of expression of *BACH2* exon 7 and 8 was confirmed with RNA-seq (III, Figure 1C).

4.2.7 Genetic complementation of BACH2, and AID expression (III)

Chicken *BACH2* was amplified from WT DT40 cDNA using primers Bach2_F and Bach2_R (Table 3), which created *Hind*III (New England BioLabs) and *Nhe*I (New England BioLabs) sites in the PCR product, respectively. After the digestion, the PCR product was cloned between *Hind*III and *Nhe*I sites of the pExpress vector (Arakawa et al., 2001), and then digested with *Spe*I (New England BioLabs). The resulting fragment (β-actin promoter-Bach2- polyA) was cloned into a pLoxPuro vector (Arakawa et al., 2001). The obtained plasmid was linearized with *Not*I and transfected into BACH2KO cells by electroporation (700V and 25 µF). Transfected cells were selected in the presence of 0.5 µg/ml puromycin (Gibco), and *BACH2* expression was verified by RT-qPCR (III, Figure 1D).

The AID transfected clones (BACH2KO/AID^{R}) were generated from the *BACH2* deleted clone (BACH2KO) by a stable transfection of AID expression construct described previously (Blagodatski et al., 2009).

4.3 Quantitative reverse transcription PCR (RT-qPCR) (I, II, III)

Total RNA was extracted from 5x10^{6} cells with RNeasy Mini Kit (Qiagen) with the optional DNase (Qiagen) treatment according to manufacturer's instructions. 1 µg of RNA was used to synthesize cDNA using qScriptTM cDNA Super Mix (Quanta Biosciences) or First Strand cDNA Synthesis Kit for RT-PCR (Roche).

Quantitative real-time PCR (RT-qPCR) was performed using SensiFastTM Sybr No-Rox Kit (Bioline) or Light Cycler FastStart DNA Master SYBR Green I Kit with Light Cycler 480 machine (Roche). The data are an average (+ SD) or (+ SEM) of at least two independent RNA extractions from separate cultures with two technical replicates. The values were compared with WT (I, II) or WT/GFP Mock (II) or WT/mC Mock (II) or Clone 18 (III), which expression was given the value 1. The expression levels of individual genes were normalized to *GAPDH* expression or in some cases to *GAPDH* and *RAB7A* expression for each cDNA sample. The obtained results were analyzed with Biogazelle qbase PLUS 2.4 software. The following primers were used (Table 4). All RT-qPCR reactions and analyses were performed following MIQE guidelines (Bustin et al., 2009).

Table 4. Primers used in RT-qPCR, ChIP-PCR and IgLV region sequence analysis (I, II, III)

Primer	Sequence (5' to 3')
GAPDH_F	GAGGTGCTGCCCAGAACATCATC
GAPDH_R	CCCGCATCAAAGGTGGAGGAAT
BACH2_F	TCATCCATGACGTTCGCCG
BACH2_R	TCACACACCAACTTGCGGAT
IgL_F	ACCACAGCGGCAGAGCAACA
IgL_R	GTGCCGTTGTGTGTGACCCT
PRDM1_F	GGCAGCCTGTCAGAATGGAAT
PRDM1_R	GCTCCTTCTTTGGGACGCTCT
RAB7A_F	GCCCCTAACACATTCAAAACC
RAB7A_R	GCTTGTGCCCGTTTTGTG
SHIP_F	GGAGTCAGGACCACCTGCCACCTG
SHIP_R	TCTTTCCGTGAGGCCTTGGGGTAGT
IRF4_F	GTATGCGCACAGCTTTGTCAAG
IRF4_R	GTTGCTCTTGTTCAAAGCACACCTCAATCT
BCL6_F	GAGAAGCCATACCCCTGTGA
BCL6_R	TGCACCTTGGTGTTTGTGAT
PAX5_F	GAACGAGTGCGATAACGACA
PAX5_R	TCGCGACCTGTTACGATAGGAT
BACH2_F	TCATCCATGACGTTCGCCG
BACH2_R	GCTCCTTCTTTGGGACGCTCT
IRF8_F	GGCACATCACCACCTCGTAT
IRF8_R	CGTGTCTGGGAACTCCTCTC
SHIP-f	GTGTCATGCTCGCTCTCTGAGCTG
SHIP-r	ATCCATGGCTGCAGCTGGAGGAAAC
IRF8-f	GGGAGAATGAGGAGAAAACCATGTTCC
IRF8-r	CTGCAAACTGCAGATCCTGCTGACAG
CVLF1	CAGTAAGCTTACCATGGCCTGGGCTCCTCTCCTCCTG
CLA2	CCTCCATTTCTAGACAGCACTTACCTGGACAGCTG

4.4 Gene expression microarray analysis (I, II)

Total RNA was prepared from $5x10^6$ cells of 2 (BCL6KO) or 3 (WT, IRF4KO, DKO) independent cultures with RNeasy Mini Kit (Qiagen) with RNase- Free DNase (Qiagen) treatment according to manufacturer's instructions. Sample preparations were performed with Agilent's Low Input Quick Amp Labeling Kit

(one-color) using 200 ng of total RNA as a starting material and samples were hybridized onto Agilent's 4x 44K Chicken V2 chip according to the manufacturer's instructions. Arrays were scanned with Agilent Technologies Scanner and numerical results extracted with Feature Extraction version 10.7.1. The data were analyzed using R/Bioconductor tool (Bioconductor version 2.7, R version 2.12.0). Data were quantile normalized, and a comprehensive quality analysis was performed. Limma package was used for the statistical testing, and cut-off values of false discovery rate below 0.01 and absolute fold change above 2 used to filter differentially expressed genes. The Venn diagrams were created by Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html). The comparisons were made based on the gene names. Hierarchical clustering of gene expression profiles was done using Euclidean distance with an average linkage. Clustering analysis was performed with R/Limma. The heat maps were generated using the GENE-E software (https://software.broadinstitute.org/GENE-E/). The microarray data has been deposited in the NCBI Gene Expression Omnibus and is accessible through GEO accession number GSE56165 (IRF4KO and WT) and GSE94836 (DKO, BCL6KO, and WT).

4.5 High-throughput RNA-sequencing (III)

Total RNA was extracted from $5x10^6$ cells of 3 independent cultures of Clone 18 and BACH2KO DT40 cells with RNeasy Mini Kit (Qiagen) with RNase-free DNase (Qiagen) treatment according to manufacturer's instructions. Libraries were prepared from 100 ng of total RNAs and subjected to deep sequencing with HiSeq2500 (Illumina) instrument using paired-end sequencing chemistry with 100 bp read length. The RNA sequencing and bioinformatic data analysis were done at The Finnish Functional Genomics Centre, Biocenter Finland, Turku. The reads were aligned against the chicken reference genome (Galgal4 assembly, downloaded from Illumina's iGenomes site) using TopHat (version 2.1.0) (Kim et al., 2013). 29 million reads (in average) were mapped with 59% of reads paired in across the different cell lines. Only uniquely aligned reads were used for the further analysis. The reads were associated with the known genes based on the Ensembl annotations, and the number of reads associated with each gene was counted using subread package (v. 1.5.0). The counts were normalized using the TMM normalization method of the edgeR R/Bioconductor package. R package Limma (Smyth, 2005) was used for performing the statistical testing between the groups. The further functional analysis was performed with Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com). The RNA-seq data have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO accession number GSE92430.

4.6 Immunoprecipitation, subcellular fractionation and Western blot analysis (I, II, III)

For immunoprecipitations with anti-BLNK and anti-PLCγ2 antibodies, and whole cell lysates (I), unstimulated and stimulated DT40 cells prior to the stimulation with 4 μg/ml monoclonal antibody M4 anti-chicken IgM (Table 5) for indicated times (1, 3, 10 or 15 min) were starved in PBS for 10 min at 40°C. Cells were lysed in RIPA lysis buffer (150 mM NaCl, 5mM EDTA (pH 8.0), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1 mM Na_3VO_4 and 1x protease inhibitor cocktail (cOmplete tablets, Roche). After removing undissolved material by centrifugation (10000xg, 10 min at 4°C), to the immunoprecipitation samples were added appropriate antibodies (Table 5) and incubated 1 hour at 4°C on the rotor. After that to the samples Protein A/G plus agarose particles (Santa Cruz Biotechnology) were added and incubated overnight at 4°C. Next day, the particles were washed three times with RIPA lysis buffer supplemented with inhibitors and denatured at 70°C for 10 min in the Lithium Dodecyl Sulfate (LDS) sample buffer (Invitrogen) supplemented with 50 mM dithiothreitol.

For the whole cell lysates (II, III), cells were lysed with RIPA buffer supplemented with 1x protease inhibitor cocktail (cOmplete tablets, Roche). Undissolved material was removed by centrifugation (10000xg, 10 min at 4°C) and samples were denatured at 70°C for 10 min in the LDS sample buffer (Invitrogen) supplemented with 50 mM dithiothreitol.

The subcellular fractions were isolated from 10^7 cells with NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (PIERCE Biotechnology) according to manufacturer's protocol. 1x protease inhibitor cocktail (cOmplete tablets, Roche) was added to the reagents CERI and NER. The concentration of proteins was measured by Bradford method using HIDEX Sense microplate reader. 20 μg and 40 μg of total proteins per sample from cytoplasmic and nuclear fractions, respectively, were loaded on the gel.

For Western blot analysis, immunoprecipitations, the whole cell lysates, and isolated fractions were separated on SDS-PAGE gels, transferred onto nitrocellulose membrane and detected by appropriate antibodies (Table 5), and chemiluminescence system (ECL, Amersham or SuperSignal West Pico, Pierce) or Odyssey FC system (Li-Cor Biosciences).

4.7 Chromatin immunoprecipitation (ChIP) (I)

A total of $3x10^6$ WT cells were cross-linked for 10 min with 1% formaldehyde, followed by quenching in a final concentration of 0.3 M glycine. After lysis in MC buffer (10 mM Tris-Cl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Igepal CA-630, pH 7.5), chromatin was sonicated using Bandelin Sonopuls in 2 ml FA lysis buffer (50 mM HEPES, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, pH 7.5) supplemented with 1x protease inhibitor cocktail (cOmplete tablets, Roche) to obtain an average length of 300 bp. Lysates were cleared by centrifugation and aliquoted into 500 µl, snap-frozen in liquid nitrogen and then diluted 1:5 with FA lysis buffer supplemented with 1x protease inhibitor cocktail (cOmplete tablets, Roche). Samples were precleared with 100 µl Protein A/G plus agarose beads (Santa Cruz Biotechnology).

Table 5. Antibodies

Antibody	Species	Description and supplier	Catalog number	Application	Used in
IRF4	Goat	M-17, Santa Cruz Biotechnology	Sc-6059	WB[a], ChIP[b]	I, II
pY	Mouse	Y99, Santa Cruz Biotechnology	Sc-7020	WB[a]	I
BCAP	Rabbit	(Okada et al., 2000)	-	WB[a]	I
PLCγ2	Rabbit	(Ishiai et al., 1999)	-	IP[c], WB[a]	I
BLNK	Rabbit	(Ishiai et al., 1999)	-	IP[c], WB[a]	I
Syk	Rabbit	(Takata et al., 1994)	-	WB[a]	I
pSYK	Rabbit	Y525/526, Cell Signaling Technology	#2711	WB[a]	I
pERK 1/2	Rabbit	T202/Y204, Cell Signaling Technology	#4370	WB[a]	I
ERK 1/2	Rabbit	137F5, Cell Signaling Technology	#4695	WB[a]	I
pPI3K	Rabbit	p85(Y458)/p55(Y199), Cell Signaling Technology	#4228	WB[a]	I
pAkt	Rabbit	S473, Cell Signaling Technology	#9271	WB[a]	I
Akt	Rabbit	Cell Signaling Technology	#9272	WB[a]	I
Chicken IgM-PE	Mouse	Clone M-1, Southern Biotechnology	8310-09	FC[d]	I, III
Chicken IgM	Mouse	Clone M-4, Southern Biotechnology	8300-01	Stimulation	I
Phalloidin-Alexa-568		A high-affinity F-actin probe, Life Technologies	A12380	IF[e]	I
anti-mouse IgG2b-Alexa-633	Goat	Life Technologies	A21146	IF[e]	I
AID	Mouse	Invitrogen	# 392.00	WB[a]	III
GAPDH	Mouse	4G5, Hytest	#5G4	WB[a]	III, II
Pax5	Mouse	A-11, Santa Cruz Biotechnology	sc-13146	WB[a]	III
PLCγ2	Rabbit	Q-20, Santa Cruz Biotechnology	sc-407	WB[a]	III
Anti-rabbit IRDye® 800CW	Goat	Li-COR Biosciences	#926-32211	WB[a]	I, III
Anti-mouse IRDye® 800CW	Goat	Li-COR Biosciences	#926-32210	WB[a]	III
Anti-goat IRDye® 800CW	Donkey	Li-COR Biosciences	#926-32214	WB[a]	II

[a)] WB, Western blot; [b)] ChIP, Chromatin immunoprecipitation; [c)] IP, immunoprecipitation; [d)] FC, flow cytometry; [e)] IF, immunofluorescence

Chromatin was immunoprecipitated with 5 µg/ml anti-IRF4 antibody (Table 5) and 5 µg of irrelevant polyclonal goat Ab as a control overnight at 4°C. After washing immunocomplexes subsequently with FA lysis buffer, FA lysis buffer with 0.5 M NaCl, and ChIP wash buffer (1 mM EDTA, 10 mM Tris-Cl, 0.25 mM LiCl, 0.5% sodium deoxycholate, 0.5% Igepal CA-630, pH 8.0), and TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), they were eluted with ChIP elution buffer (10 mM EDTA, 50 mM Tris-Cl, 1% SDS, pH 7.5). To reverse cross-linked samples, the SDS was adjusted to 0.5%, and the remaining RNA and proteins were digested using RNase A and pronase (Roche) at 42°C for 2h and incubated overnight at 65°C. DNA-containing samples were purified with QIAquick PCR purification kit (Qiagen). Samples were analyzed by ChIP-PCR. 5 µl of ChIP DNA template was amplified in 32 cycles with Phusion polymerase (Finnzymes) in "GC" buffer with 3.5% DMSO. The promoter region of *INPP5D* was amplified with primers SHIP-f and SHIP-r, and promoter region of *IRF8* with primers IRF8-f and IRF8-r (Table 4). The control chromatin region of *GAPDH* was amplified with primers GAPDH_F and GAPDH_R. ChIP experiments were performed five times.

4.8 Calcium flux measurement (I)

The calcium measurements in DT40 cells were performed as described in (Stork et al., 2004). Cells were loaded with 1 µM Fluo4 AM (Molecular Probes) with 0.015 % Pluronic F127 and stimulated with 1 µg/ml anti-chicken IgM M4 Ab (Table 5). Changes in the fluorescence intensity were monitored by using FACS Calibur cytometer (BD). Equal loading of samples was controlled by treatment with 100 nM ionomycin (Sigma). The kinetics overlay was performed with FlowJo software (version 9.7.1; ThreeStar Inc.).

4.9 Microscopy (I)

For confocal and Total Internal Reflection Fluorescence (TIRF) microscopy multiwell microscopy slides or chambered coverslips, respectively, were coated with 5 µg/ml anti-chicken IgM (M4) in PBS. Cells in imaging buffer (PBS, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 1 g/L D-glucose, 0.5 % FCS), were let to settle on slides for 20 min at 37°C in CO_2 incubator and fixed with 4 % formaldehyde. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and stained with Phalloidin Alexa-568. Samples were mounted in Fluoromount-G for confocal or PBS for TIRF microscopy. Confocal images were acquired using Zeiss LSM510, equipped with Plan-Apochromat 63x/1.40 Oil DIC M27 objective, or Zeiss TIRF

microscope, equipped with alpha Plan-Apochromat 100x/1.46 Oil DIC (UV) objective and ORCA-Flash 4.0 CMOS digital camera (Hamamatsu) (Cell Imaging Core, Turku Centre for Biotechnology). Data processing and analysis were done in FIJI (http://fiji.sc/Fiji). For quantification of F-actin intensity, binary images were generated with blind manual thresholding to create regions of interest corresponding to F-actin staining area of the cells. The intensity within regions of interest was measured from 17-21 cells per cell line.

4.10 ELISA analysis of IgM production (II)

For the ELISA assay, 10^6 cells were grown in 1 ml of DT40 medium without chicken serum for 24h in humidified atmosphere with 5% CO_2 at +40°C. The medium containing secreted IgM was collected from two independent cultures (two biological repeats) of each cell line and used in a dilution of 1:10 or 1:100 in duplicates for the analysis with the chicken IgM ELISA kit (E33-102, Bethyl Laboratories, Inc.) according to manufacturer's instructions. The absorbance was measured by an ELISA plate reader (Multiscan EX, Thermo Electron Corporation) at 450 nm and the amount of chicken IgM in unknown samples was calculated from a standard curve with curve fitting software GraphPad Prism version 7.01 (GraphPad Software).

4.11 Analysis of cellular growth (III)

Clone 18, BACH2KO and BACH2KO/BACH2^R cell cultures were diluted to 10^4 cell/ml and the density of cultures was measured at 24 h intervals with Automated Cell Counter TC20 (Bio-Rad) in the presence of Trypan Blue Dye (Bio-Rad).

4.12 Measurement of cell proliferation (III)

To study the proliferation of cells, Clone 18, BACH2KO and BACH2KO/BACH2R cells were washed twice with PBS and re-suspended in 0.5 μM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) in 5% FCS/PBS (weight/volume) and incubated for 10 min at RT. The labeling was stopped with the 10x volume of 5% FCS/PBS (weight/volume), and cells were washed twice with 5% FCS/PBS. CFSE labeled cells were cultured for 72 hours. The CFSE staining of the cells was measured and analyzed with BD AccuriC6 system. The CFSE dye is diluted to half with each cell division and the reduction in the mean fluorescence intensity of CFSE during time course shows the speed of cell divi-

sions in tested cell lines. The linear regression analysis was performed with GraphPad Prism version 7.01(GraphPad Software).

4.13 Surface IgM gain assay and IgLV region sequence analysis (III)

Cells were stained with anti-chicken-IgM-PE Ab (Table 5), and IgM negative sub-clones were sorted by MACS (Miltenyi Biotec) or by flow cytometric single-cell sorting (FACSAria IIu Cell Sorter, BD). 20-30 subclones per cell line were cultured for 14-41 days and stained with anti-chicken-IgM-PE Ab (Table 5) before each FACS analysis. FACS analyses were performed with FACSCalibur (BD) and BD AccuriC6. For the *IgLV* region sequence analysis, DNA was isolated from 23 and 21 sub-clones from Clone18 and BACH2KO, respectively, at day 41 with MagnaPure 96 DNA and Viral NA Large Volume Kit (Roche). The VJ region was amplified with primers CVLF1 and CLA2 (VL52) using Phusion high-fidelity polymerase (Finnzymes) for 30 cycles. Products were extracted from the agarose gel with GeneJet Gel Extraction Kit (ThermoFisher Scientific) and cloned into pCR-Blunt II-TOPO with Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Approximately ten mini preps per subclone were sequenced with the primer CVLF1 by targeted sequencing at Finnish Functional Genomics Centre, Biocenter Finland, Turku. Sequence alignments were performed with MultAlin (version 5.4.1) (Corpet, 1988) by simultaneously comparing all the sequences from an individual sub-clone. Sequence analysis was performed as previously described (Kothapalli and Fugmann, 2011; Sale et al., 2001). Briefly, a mutation was classified as an Ig gene conversion when ≥8bp containing mutated sequence matched to the pseudo-V elements in the NCBI BLASTN program (http://blast.ncbi.nlm.nih.gov) (with settings “Database: nucleotide collection nr/nt” of “Organism: Gallusgallus” and “Entrez query: pseudo”). When mutation did not match any pseudo-V sequence was classified as SHM if only one base pair was changed, as deletion if one or more base pairs were missing and as insertion, if base pairs were inserted (a duplication of nearby sequence).

4.14 Statistical analyses (I, II, III)

Statistical analyses were performed using GraphPad Prism version 7.01 for Mac (GraphPad Software). The statistical significance of changes in the gene expression (RT-qPCR), protein expression (Western blot quantification), actin polymerization experiment, ELISA assay, ChIP analysis and mutation frequencies were determined by two-tailed Student’s t-test (when two groups

were compared) or one-way ANOVA with Holm-Sidak's post-test (when more than two groups were compared). The non-parametric tests: Kruskal-Wallis with Dunn's post-test (three groups) and two-tailed Mann-Whitney test (two groups) were used to test statistical significance in the sIgM gain assay.

5 RESULTS AND DISCUSSION

5.1 Molecular pathways and target genes regulated by IRF4 (I, II)

To investigate the role of IRF4 in B cells, I disrupted the *IRF4* gene in DT40 B cells by homologous recombination. The targeting constructs were designed to remove exons 2 and 3 encoding DNA binding domain of IRF4. After targeting the lack of IRF4 protein was confirmed by Western blot analysis with the anti-IRF4 antibody (I, Figure 1). IRF4 knockout (IRF4KO) and wild-type (WT) cells were subjected to the transcriptional profiling by Agilent 4 x 44K Chicken V2 chip microarray.

The analysis of microarray data with the Ingenuity Pathway Analysis revealed that among 2793 genes (absolute fold change ≥ 2 and $p \leq 0.05$) affected by IRF4 deficiency were genes encoding: transcription factors with role in B cell differentiation such as *PRDM1* (encoding BLIMP1), *ID3*, *ID2*, *STAT1*, *CIITA* (MHC II Transactivator), *SPI1* (known as PU.1, and IRF4 cofactor) and *IRF8*; enzymes important for Ig diversification processes *AICDA* (encoding AID), *POLQ* and *RAG2*, transmembrane receptors crucial for B-T cell interaction and activation *CD40* and *IL21R*, as well as proteins involved in BCR signaling pathway *SYK*, *BLNK*, *PTPN6* (encoding SHP1), and *PIK3AP1* (encoding BCAP) (I, Figure S1 and Table S1; II, Figure 2B, Figure 12). The gene expression profiling study showed that IRF4 is involved (directly or indirectly) in a wide spectrum of B cell functions. The results verified reported functions for IRF4, such as promotion of plasma cell differentiation and CSR by direct upregulation of *PRDM1* and *AICDA*, respectively (Ochiai et al., 2013; Sciammas et al., 2006). The IRF4-PU.1-E47 complex induces MHC II transactivator promoter III (CIITA-PIII) (van der Stoep et al., 2004). However, the role of IRF4 in the regulation of BCR signaling pathways has not been previously reported. And B cell-specific transcription factors including PAX5, BCL6, AIOLOS, IKAROS and HELIOS can influence the BCR signaling strength and response (Alinikula et al., 2010; Alinikula et al., 2011; Narvi et al., 2007; Nera et al., 2006a; Nera et al., 2006b).

5.1.1 IRF4 directly represses IRF8 in DT40 B cells (unpublished results)

Another interesting finding arose from the notion that in the absence of *IRF4* the expression of *IRF8*—a very closely related IRF family member—was increased significantly (II, Figure 2). In early B cell development IRF4 and IRF8 display redundant functions (Shukla and Lu, 2014), however, in late B cell development,

their expression (Figure 7) and their roles seem to be reciprocal. Xu et al. (Xu et al., 2015) have shown that IRF8 can directly repress *IRF4* in activated B cells, but although overexpression of IRF4 in these cells resulted in a lower expression of IRF8, IRF4 seemed not to bind to *IRF8* locus (in mouse). However, IRF4 binding sites to *IRF8* gene are found in human GM12878 B-lymphocyte cell line [human ENCODE database (ENCODE project consortium)].

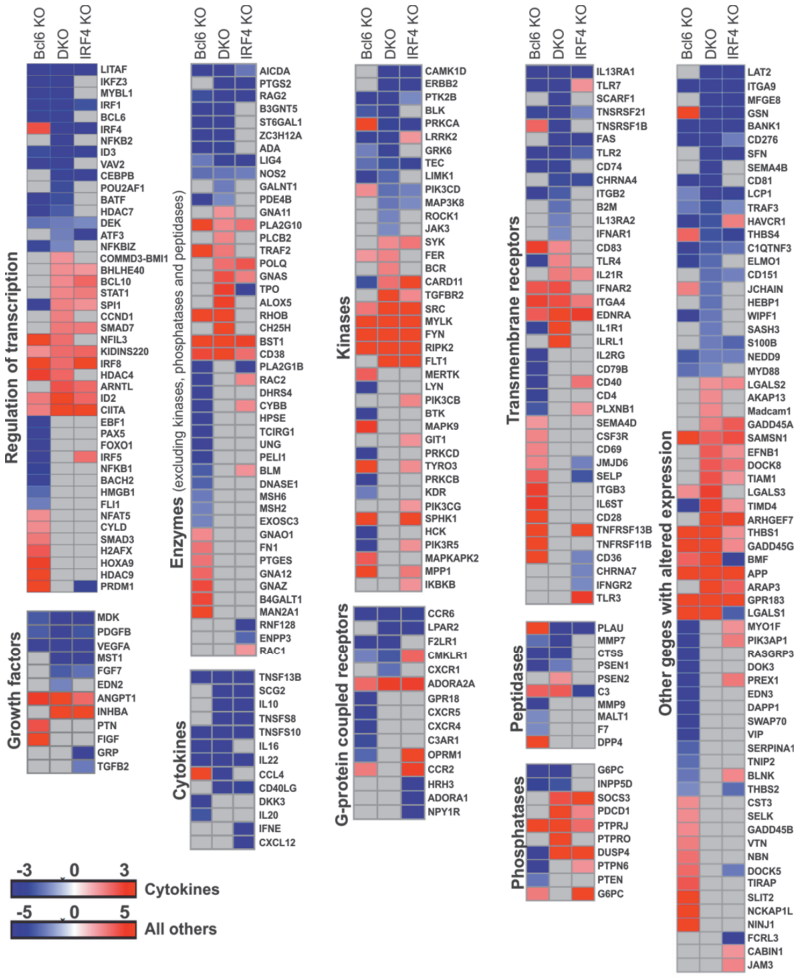

Figure 12. Gene expression profiles of IRF4 knockout (IRF4KO), double knockout of IRF4 and BCL6 (DKO), and BCL6 knockout (BCL6KO) cells relative to WT cells with absolute fold change ≥ 2 and p ≤0.01

Colors are representing fold change, and gray color indicates no change within settled threshold. The heat maps were generated using the GENE-E software by Minna Kyläniemi.

To validate the effect of IRF4 deficiency on increased IRF8 expression, the mRNA levels of *IRF8* were measured by RT-qPCR in WT, IRF4KO and IRF4KO cells restored with IRF4 (IRF4KO/IRF4). The IRF4 restored cell line

expressed ~50% of the IRF4 protein level in WT cells (I, Figure 1D). The RT-qPCR confirmed the 2-fold increase of *IRF8* expression in IRF4KO cells and re-expression of IRF4 restored it to the WT level (Figure 13A). To test whether IRF4 represses *IRF8* directly, I performed ChIP with anti-IRF4 antibody and ChIP-PCR for *IRF8* promoter region. The experiments indicated that IRF4 bound to this region *in vivo* (Figure 13B).

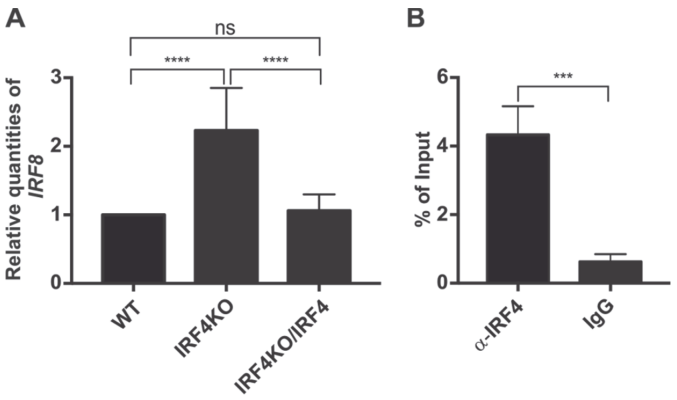

Figure 13. IRF4 directly suppresses IRF8 expression
(A) The mRNA levels of IRF8 in WT, IRF4KO and IRF4KO/IRF4 cell lines; quantified by RT-qPCR and normalized to *GAPDH*, shown in comparison with WT level, set as 1. (B) IRF4 binding to the *IRF8* promoter region examined by ChIP with anti-IRF4 antibody (α-IRF4) and unrelated polyclonal goat antibody (IgG) used as a control. Input and immunoprecipitated DNA were amplified by PCR with primers specific for the promoter region of *IRF8*. The PCR products were quantified and presented as % of input. Mean + SD of 6 (A) or 5 (B) independent experiments. ****, p<0.0001; ***, p=0.0001; ns, not statistically significant. (A) One-way ANOVA with Holm-Sidak's post-test or (B) two-tailed Student's t-test.

The IRF4KO DT40 data is supported by Xu et al. (Xu et al., 2015) finding that IRF4 and IRF8 counter-regulate each other to regulate opposite cellular states. High expression of IRF4 has been shown to promote plasma cell differentiation (Ochiai et al., 2013; Sciammas et al., 2006) whereas IRF8 together with PU.1 are antagonizing it to allow GC response (Carotta et al., 2014). In DT40 cells the expression of *CD40*, *IL21R,* and *CIITA* were increased in the absence of IRF4 with a simultaneous increase of *IRF8* expression (Figure 12). These findings are indicating that IRF8 is preparing B cells for antigen presentation to T_{FH} cells and allowing them for affinity maturation. Of note, the expression of *CD40* and *IL21R* are elevated in activated B cells with a high IRF8 expression as well (Xu et al., 2015). The CD40 receptor is critical for a germinal center response (Kawabe et al., 1994) and CD40 signaling has been shown to modulate the outcome of B cell differentiation by miR-125b-mediated inhibition of BLIMP1 (Basu et al., 2016). The CD40 ligation during B cell activation inhibits plasma cell differentiation and enhances memory B cell commitment (Raman et al., 2003; Randall et al., 1998). Thus, the elevated expression of *CD40* in IRF4KO

DT40 cells might serve as an additional mechanism preventing premature B cell differentiation into antibody-secreting plasma cells.

5.1.2 Impaired activation of SYK and BLNK results in an attenuated ERK phosphorylation in the absence of IRF4 (I)

Microarray analysis of IRF4KO cells showed enrichment of differentially expressed genes with the function involved in the BCR signaling. Given that B cell fate decisions including proliferation, survival, and antigen activation critically depend on the BCR signaling (Casola et al., 2004; Kitamura et al., 1991; Kraus et al., 2004; Meffre and Nussenzweig, 2002) and since the role of IRF4 in the regulation of BCR signaling has not been previously assessed, we decided to investigate it more thoroughly.

One of the earliest BCR signaling events upon antigen encounter or BCR-crosslinking is recruitment and the phosphorylation of kinases such as spleen tyrosine kinase (SYK) to the ITAMs of Igα and Igβ. Phosphorylated SYK (pSYK) then activates BLNK, BTK, VAV, and BCAP by phosphorylation thereby triggering a downstream signaling cascade resulting in Ca^{2+} flux and activation of ERK and AKT pathways in B cells (Baba and Kurosaki, 2011; Kurosaki, 2002; Kurosaki et al., 2010). To determine the BCR signal strength, the phosphorylation status of SYK, BLNK, and ERK were measured before and after BCR-crosslinking with anti-chicken IgM antibodies. Although SYK and BLNK mRNA (I, Figure S1), as well as the protein levels (I, Figure S2), were upregulated in IRF4KO cells, these cells displayed a weaker activation of SYK, BLNK, and ERK in the response to the BCR stimulation than their WT counterparts (I, Figure 3A and B).

The attenuated activation of SYK and BLNK in IRF4KO cells correlates with the enhanced expression of IRF8 in these cells (Figure 12). Recently Xu et al. showed that IRF8 was responsible for dampening BCR signaling in B cells, as *Irf8*$^{-/-}$ mice more frequently produced BCR clones of low-affinity upon immunization due to a greater BCR-induced phosphorylation of SYK and BLNK in naïve and GC B cells than did their IRF8-sufficient counterparts (Xu et al., 2015). Disruption of *Irf8* increased the expression of IRF4 (Xu et al., 2015) so the effect of lowered BCR signaling treshold might result from the elevated expression of IRF4. The findings from IRF4KO DT40 cells are in accordance with another study showing that GC centroblasts, which are characterized by high IRF8 and diminished IRF4 expression, have the same signaling profile of low pSYK, pBLNK and pERK (Khalil et al., 2012) as IRF4KO cells.

Interestingly, activation of ERK signaling is critical for the upregulation of BLIMP1 and differentiation of GC B cells into plasma cells (Yasuda et al., 2011). Given that IRF4 acts in a dose-dependent manner where low levels of IRF4 activate *BCL6* expression, and high levels promote BLIPM1 expression (Ochiai et al., 2013), it is not surprising that low levels of IRF4 observed in our IRF4KO/IRF4 cells are insufficient to restore ERK signaling required for BLIMP1 upregulation and plasma cell program initiation (Yasuda et al., 2011). Taken together, the suppression of IRF4 expression upon antigen activation, as observed in GCs, is required under physiological conditions for enhanced IRF8 expression and increased BCR signaling threshold for ERK activation in order to achieve proper affinity maturation of B cells before terminal differentiation into a plasma cell.

5.1.3 IRF4KO cells have compromised BCR-mediated actin polymerization response to the surface-bound antigen (I)

Given that binding of antigens to BCRs trigger activation of multiple actin-regulating proteins and thus actin polymerization coordinates immunological synapse formation (Harwood and Batista, 2011), it was asked whether compromised activation of SYK in IRF4KO cells affected actin polymerization too. The induction of actin polymerization was tested by immobilized antigen (microscopy glass coated with anti-chicken IgM antibodies) to resemble antigen presentation by dendritic cells and macrophages. To investigate actin polymerization the confocal microscopy, as well as TIRF microscopy that provides higher resolution imaging were used. Visualization with fluorescent phalloidin showed a striking defect in actin polymerization to the surface-bound antigen in IRF4KO cells when compared with WT cells (I, Figure 4).

Table 6. KEGG enrichment analysis for ranked list of genes differentially expressed in IRF4KO cells vs. WT cells.

KEGG.ID	Term	Annotated	*p*-value
4510	Focal adhesion	160	0.000753
4910	Insulin signaling pathway	101	0.00753
4120	Ubiquitin-mediated proteolysis	108	0.0154
4530	Tight junction	99	0.0154
4150	mTOR signaling pathway	39	0.0166
4810	Regulation of actin cytoskeleton	159	0.0386

The hypomorphic IRF4 expression in IRF4KO/IRF4 cells was unable to restore the impairment of actin polymerization even though the attenuation of SYK phosphorylation upon BCR-crosslinking was restored to the WT levels in these cells (I, Figure 3A). However, there weren't any known genes related to the cytoskeletal functions that would have been highly deregulated in the absence of

IRF4 (microarray dataset deposited at GEO). The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis for ranked list of genes was used to search enrichment of actin-related pathways. This analysis does not use predetermined thresholds like certain p-value of fold-change. The KEGG analysis showed enrichment in the pathways for focal adhesion and regulation of actin cytoskeleton (Table 6). Thus, the defect in BCR-induced actin polymerization might result from the additive effect of small gene expression changes in these pathways.

To fully exclude the BCR signaling as a cause of the defect, additional experiments could be performed. The BCR signaling could be functionally bypassed by using PMA/ionomycin or pervanadate. These drugs act downstream of the antigen receptor and activate cells through diverse pathways allowing to overcome the SYK and BLNK that have been affected in these IRF4KO cells. One would expect that cytoskeletal functions would be restored in the presence of the drugs if the BCR signaling is the source for the defect. Alternatively, if cytoskeletal rearrangements continue to be affected, it would imply that BCR signaling is not the basis and the cumulative effect of gene expression changes is at play. However, the power of the additional experiments is questionable since the hypomorphic IRF4 expression in IRF4KO/IRF4 cells did not restore the cytoskeletal defect that could arise independently from *IRF4* targeting.

5.1.4 Disruption of IRF4 augments PI3K/AKT signaling (I)

The gene expression comparison between WT and IRF4KO cells (I, Figure S1 and Table S1) revealed that IRF4KO cells had increased expression of *B cell adaptor protein* (*BCAP*) encoded by *Phosphoinositide-3-Kinase Adaptor Protein 1* (*PIK3AP1*). As the gene name indicates, BCAP is an adaptor protein for Phosphoinositide-3-Kinase (PI3K). Tyrosine phosphorylation of BCAP by SYK and BTK provides the binding site(s) for PI3K (Okada et al., 2000). In turn, activated PI3K catalyses phosphorylation of phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) into phosphatidylinositol-3,4,5-trisphosphate (PIP_3) which recruits PLCγ2, AKT, BTK and PDK1 to the plasma membrane (Falasca et al., 1998; Franke et al., 1997) (Bolland et al., 1998) (Anderson et al., 1998). PI3K consists of a catalytic p110 subunit and of p50, p55 or p85 regulatory subunit. Activation of membrane-bound AKT is achieved through phosphorylation by PDK1 at threonine 308 (T308) and by mTOR complex 2 at serine 473 (S473) (Alessi et al., 1997; Sarbassov et al., 2005).

To evaluate the impact of IRF4 disruption on PI3K/AKT signal transduction, I checked the activation status of BCAP, PI3K p55 and AKT by Western blotting before and after BCR-crosslinking with anti-chicken IgM antibodies (I, Figure

5). In IRF4KO cells the expression of short BCAP isoform before stimulation was 2-fold higher than in WT and IRF4KO/IRF4 cells, validating the mRNA level of BCAP seen in the microarray (I, Figure S1 and Table S1). Upon BCR engagement, the phosphorylation of BCAP (observed as a shift in the migration on the gel), as well as tyrosine 199 (Y199) of PI3K p55 and S473 of AKT, was enhanced in the absence of IRF4 and partially restored in hypomorphic IRF4KO/IRF4 cells to the WT level (I, Figure 5). The results indicated that regulation of BCAP expression by IRF4 is reflected in the activity of PI3K/AKT signaling pathway. Given that activity of PI3K is required for antigen-induced robust proliferation and potentiates *BCL6* expression (Zhang et al., 2012) the suppression of *IRF4* expression upon activation seems to be a prerequisite for B cell affinity maturation. Further experiments are needed to resolve the exact role of IRF4 in B cell development and function.

5.1.5 IRF4-deficient cells display elevated Ca^{2+} release from endoplasmic reticulum likely due to lower expression of SHIP (I)

IRF4 deficiency resulted in the changed BCR signal transduction to the two major pathways, ERK and AKT. Components of early BCR signal mediators SYK, BLNK, PLCγ2, BCAP, BTK, and PI3K, are known to influence BCR-induced Ca^{2+} flux (Falasca et al., 1998; Ishiai et al., 1999; Takata and Kurosaki, 1996; Takata et al., 1994; Yamazaki et al., 2002). The Ca^{2+} flux controls lymphocytes activation, differentiation, apoptosis and effector function [reviewed in (Baba and Kurosaki, 2011)]. Given that decreased phosphorylation of SYK and BLNK, unaffected phosphorylation of PLCγ2, and enhanced phosphorylation of BCAP and PI3K is found in IRF4KO cells (I, Figure 3 and 5), I was prompted to check how these seemingly opposite changes related to the Ca^{2+} flux.

The lack of IRF4 resulted in an increase of BCR-induced Ca^{2+} flux by approximately 40% when compared with WT cells (I, Figure 1A). In the hypomorphic IRF4KO/IRF4 cell line, the Ca^{2+} mobilization was proportionally restored to the IRF4 expression level (which is 50% of the WT level). It is important to note that IRF4KO/IRF4 cell line had the largest variability in BCR-induced Ca^{2+} response. IRF4KO cell line always had higher BCR-induced Ca^{2+} flux than WT cells, but the IRF4KO/IRF4 cell line in some experimental repeats had exactly WT level, and in some, the level was between IRF4KO and WT level.

Given the crucial role of BLNK in the recruitment to the membrane and activation of PLCγ2 that mediates Ca^{2+} flux (Engelke et al., 2013), it was surprising that enhanced Ca^{2+} mobilization occurred in the absence of IRF4. To understand this phenomenon, I carefully checked the expression of genes regulating Ca^{2+}

flux. Indeed, the expression of Inositol Polyphospate-5-Posphateses *INPP5B*, *INPP5F* were strongly reduced in IRF4KO cells as seen in the microarray (I, Table S1), and *INPP5D* (encoding SHIP) verified by RT-qPCR (I, Figure 6A).

SHIP is a negative regulator of BCR signaling and Ca^{2+} flux (Bolland et al., 1998; Liu et al., 1998; Okada et al., 1998) that maintains the balance of plasma membrane phospholipids (Figure 14). Since IRF4 re-expression in IRF4KO/IRF4 cells fully restored *INPP5D* expression to the WT level and that IRF4 is bound to the promoter region of *INPP5D* gene-encoding SHIP (I, Figure 6) it was concluded that IRF4 is a likely directly regulator of SHIP expression. Furthermore, 2-fold reduced expression of SHIP in IRF4KO cells may lead to an imbalance in the PIP_3 metabolism and thus results in the enhanced BCR-induced Ca^{2+} mobilization (Figure 14) as SHIP-deficient DT40 cells display increased calcium response (Okada et al., 1998). For further experimental testing of this model a measurement of different phosphatidylinositol phosphates concentrations upon BCR ligation in the absence and presence of IRF4 would be required. However, the exact measurement of plasma membrane phospholipids with the demarcation of various classes upon BCR ligation is very challenging due to the absence of chicken specific antibodies.

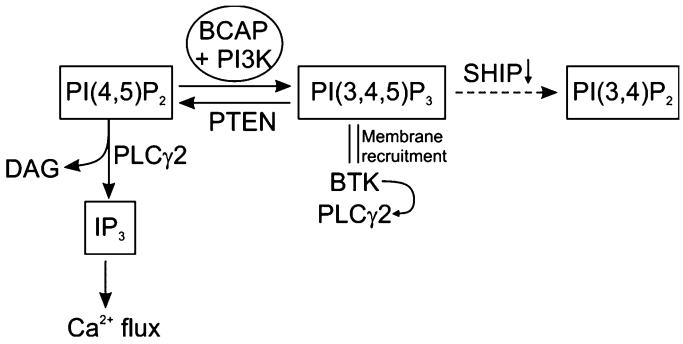

Figure 14. Model of regulation of plasma membrane phospholipids in the absence of IRF4

Phosphorylated by SYK, BCAP attracts to the plasma membrane PI3K which catalyzes phosphorylation of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3). The PIP3 mediates membrane recruitment and coactivation of BTK and PLC 2. Activated PLC 2 by SYK and BTK cleaves PI(4,5)P2 into diacylglycerol (DAG) and inositol triphosphate (IP3) which in turn binds to the IP3 receptors in the endoplasmic reticulum membrane causing rapid Ca2+ flux. SHIP dephosphorylates PIP3 into phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2), thereby withdrawing the PIP3 pool from the reaction and thus preventing BTK membrane association. In the absence of IRF4, the expression of SHIP is reduced, resulting in a changed balance of phospholipids and therefore increased Ca2+ flux. BCAP, B-cell adaptor for PI3K; PI3K, Phosphoinositide 3-kinase; PTEN, Phosphatase and tensin homolog; PLC 2, Phospholipase C 2; BTK, Bruton's tyrosine kinase; SHIP, SH2-containing inositol phosphatase; SYK, Spleen tyrosine kinase.

The positive regulation of SHIP expression by IRF4 can be crucial for proper B cell activation and generation of high-affinity B cells, as SHIP-deficient mice had a lowered antigen-activation threshold leading to the spontaneous generation of GCs and increased CSR (Helgason et al., 2000; Liu et al., 1998). Furthermore, conditional knockout of SHIP in murine GC B cells demonstrates that despite normal SHM rate these B cells have low-affinity BCRs of due to a defect in the T cell-dependent selection of B cells (Leung et al., 2009). Hence the regulation of appropriate levels of SHIP and thus inhibition of BCR signaling is critical for the generation of high-affinity antibodies.

The direct regulation of SHIP by IRF4 may not be restricted only to DT40 B cell line as IRF4 binding sites to the *INPP5D* gene are found in human GM12878 B-lymphocyte cell line [human ENCODE database (Bernstein et al., 2012)] and activated murine B cells (48h after LPS stimulation) [IRF4 ChIP-seq dataset (Xu et al., 2015)]. Interestingly, IRF8 binds to the *INPP5D* gene at the same position as IRF4 [IRF8 ChIP-seq dataset from (Xu et al., 2015)] in murine GC B cells. However, further work is required to understand the nature of these bindings and the role of IRF4 and IRF8 in the regulation of SHIP expression in primary B cells.

5.1.6 BCL6 inactivation in IRF4KO cells cannot induce plasma cell differentiation and antibody secretion in contrast to single BCL6 knockout cells (II)

Disruption of *BCL6* in DT40 B cells leads to upregulation of *PRDM1* mRNA and the functional outcome of plasmacytic differentiation characterized by IgM secretion (Alinikula et al., 2011). I inactivated *BCL6* in IRF4KO cells to elucidate the role of IRF4 in plasma cell differentiation and antibody secretion. The lack of *IRF4* and *BCL6* expression in *IRF4*$^{-/-}$/*BCL6*$^{-/-}$ double knockout cells (DKO) was verified by Western blot and RT-qPCR, respectively (II, Figure 1A and B).

The DKO cells did not secrete IgM (II, Figure 1C). This is analogous to IRF4KO cells but in a contrast to the single *BCL6* knockout cells (BCL6KO) that secreted high levels of IgM when compared with WT cells (II, Figure 1C). This result indicates that removal of *BCL6* is insufficient for induction of plasma cell differentiation in the absence of IRF4. This finding is in line with earlier observations that IRF4 is critical for plasma cell differentiation (Klein et al., 2006; Mittrucker et al., 1997; Ochiai et al., 2013; Willis et al., 2014).

To check what gene expression changes deletion of *BCL6* in IRF4KO cells induced, the RT-qPCRs and transcriptional profiling of WT, BCL6KO, IRF4KO

and DKO DT40 B cells on Agilent 4 x 44K chicken V2 chip microarray were performed. The expression of *PRDM1* (encoding a plasma cell master regulator BLIMP1) was neither upregulated in IRF4KO nor DKO cells (Figure 12, II, Figure 1D, and Figure 2) thus most likely causing the lack of antibody secretion. The deletion of *IRF4* caused an upregulation of *BACH2*, *PAX5,* and *IRF8* expression, whereas the deletion of both *IRF4* and *BCL6* caused a reduction of *BACH2* expression by 50%, left *IRF8* expression upregulated by 100% and *PAX5* expression unchanged when compared with WT cells (II, Figure 1D). Furthermore, the expression of other transcription factors regulating the B cell fate was upregulated or unaltered in DKO cells. These include PU.1 (encoded by *SPI1*), and EBF1 (Figure 12 and II, Figure 2B) that could be responsible for poised differentiation into antibody-secreting plasma cells, as all of them negatively regulate plasma cell differentiation (Carotta et al., 2014; Kikuchi et al., 2012).

Xu et al. showed that in activated B cells IRF4 in high doses inhibits *IRF8* expression and also directly represses *BACH2*, *SPIB,* and *EBF1* (Xu et al., 2015). Therefore, IRF4 function in terminal B cell differentiation may not be limited to the upregulation of *PRDM1* expression but may contribute to turning off the B cell gene expression program as well.

These results also indicate that IRF4 can repress *BACH2* (increased expression of *BACH2* in the absence of IRF4), and that BCL6 is a major factor for maintaining *BACH2* expression, as in cells deficient in *BCL6* (BCL6KO and DKO) *BACH2* expression drops dramatically and independently of BLIMP1 action (DKO cells did not express BLIMP1) (II, Figure 1D). The obtained results are in line with another study showing that BCL6 increases BACH2 expression and stability (Huang et al., 2014), additionally evidencing that similar regulation occurs in DT40 B cells.

5.1.7 Ectopic expression of BLIMP1 can induce IgM secretion only in the presence of IRF4 (II)

The ectopic BLIMP1 expression can induce Ig secretion (Kallies et al., 2007; Turner et al., 1994). To test whether the lack of BLIMP1 upregulation in IRF4 deficient cells alone is responsible for the absence of antibody secretion, I overexpressed *BLIMP1* in WT, IRF4KO and DKO cells from lentiviral constructs (II, Figure 3A and B) and measured IgM secretion. The introduced BLIMP1 overexpression only increased IgM secretion in WT cells (WT/B1-GFP) (II, Figure 3C). IRF4KO/B1-GFP and DKO/B1-GFP cells, despite higher expression of BLIMP1 than WT/B1-GFP cells, did not start secreting IgM (II,

Figure 3B, and C). This indicates that the presence of IRF4 is required for the secretion.

To reveal what changes the overexpression of BLIMP1 imposed on another transcription factors such as BCL6, PAX5, and IRF4, their expression by RT-qPCR was measured. Surprisingly overexpressed BLIMP1 did not have any impact on PAX5 expression in any of tested cell lines (WT/B1-GFP, IRF4KO/B1-GFP, and DKO/B1-GFP). It caused reduced expression of IRF4 in antibody secreting WT/B1-GFP cells (II, Figure 3D). Previously, it has been suggested that BLIMP1 represses PAX5 directly (Lin et al., 2002); however, in some models, ectopic expression of BLIMP1 did not silence *PAX5* (Sciammas and Davis, 2004) and the early phase of *PAX5* downregulation was independent of BLIMP1 (Kallies et al., 2007). Further experiments are required to understand on a molecular level how ectopic BLIMP1 induces IgM secretion which was observed only in the presence of IRF4 (WT cells) (Figure 15).

5.1.8 Re-expression of IRF4 in DKO cells induces antibody secretion (II)

To verify the role of IRF4 in the induction of antibody secretion, I performed lentiviral overexpression of IRF4 in WT, IRF4KO and DKO cells (II, Figure 4A and B). I then determined the amount of secreted IgM from IRF4 transduced cell lines. IRF4 transduction induced moderate IgM secretion in IRF4KO (IRF4KO/IRF4^{mC}) cells whereas in DKO (DKO/IRF4^{mC}) cells it induces a strong secretion (II, Figure 4C) even though DKO/IRF4^{mC} cells expressed much less IRF4 (on protein and mRNA level) than WT/IRF4^{mC} and IRF4KO/IRF4^{mC} counterparts (II, Figure 4B and D).

Next, to check whether IRF4 transduction affected the expression of *PRDM1*, *BCL6,* and *PAX5*, RT-qPCRs were performed for these transcription factors. *IRF4* overexpression in WT and IRF4KO cells did not increase *PRDM1* expression but rather increased *BCL6* expression (II, Figure 4D). This is reminiscent of IRF4-mediated activation of *BCL6* observed under low levels of IRF4 in the initiation of germinal center reaction [described in "IRF4 graded expression model", (Ochiai et al., 2013; Sciammas et al., 2011; Sciammas et al., 2006)].

Thus, the question arises whether more than 25-fold overexpression of IRF4 in WT and in IRF4KO DT40 cells (II, Figure 4D) really represents a high expression of *IRF4*. Because in accordance with "IRF4 graded expression model" a high expression of IRF4 should change IRF4 binding properties from EICE to ISRE motif to induce *PRDM1* expression (Ochiai et al., 2013; Sciammas

et al., 2011; Sciammas et al., 2006). IRF4 binds together with PU.1 or SPIB to EICE motif. The expression of PU.1 encoded by *SPI1* is strongly downregulated in antibody-secreting BCL6KO cells (Figure 12). Therefore, is the expression of *SPI1* playing a major role in the switch of IRF4 function toward the promotion of plasma cell differentiation? To reveal it further experiments are required.

In accordance with IgM secretion, reduced *PAX5* and enhanced *PRDM1* expression was only observed in the DKO cells with enforced *IRF4* expression. Therefore re-expression of IRF4 in DKO cells restored the phenotype of single *BCL6* knockout cells [(Alinikula et al., 2011) and (II, Figure 1)], indicating once again that IRF4 together with BLIMP1 is required for antibody secretion and that IRF4 cannot induce robust secretion in the presence of BCL6 (Figure 15).

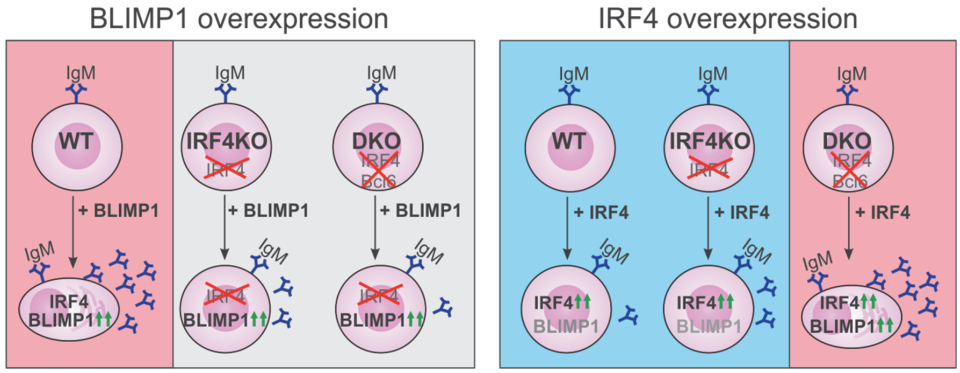

Figure 15. Induction of IgM secretion in DT40 cell lines by BLIMP1 or IRF4 overexpression

WT, wild-type; IRF4KO, *IRF4* knockout; DKO, double knockout of *IRF4* and *BCL6*. IgM secretion can be induced by ectopic overexpression of BLIMP1 only in the presence of IRF4, or by overexpression of IRF4 when BLIMP1 is de-repressed in the absence of *BCL6* (situations indicated by reddish background).

5.2 BACH2 regulates generation of mutations in Ig locus (III)

To examine the role of BACH2 in the generation of mutations in immunoglobulin locus, I deleted the DNA binding domain of *BACH2* in B cells undergoing Ig mutations. I used a variant of DT40 cell line (Clone 18) that is commonly used for GCV and SHM studies (see *Review of the literature: Immunoglobulin gene conversion*). I deleted the coding parts of the exons 7 and 8 of *BACH2* gene yielding a *BACH2* knockout (BACH2KO) cell line. The removal of *BACH2* exon 7 and 8 was verified by genomic PCR, RT-qPCR, and RNA-seq (III, Figure 1).

BACH2KO cells had exhibited a lowered mutation frequency. *BACH2* gene inactivation strongly reduced the gain of surface Immunoglobulin M (sIgM) expres-

sion in an sIgM gain assay when compared with Clone 18 cells (III, Figure 2A). The proliferation rate was not affected (III, Figure S1). This assay measures the frequency of mutation events in the immunoglobulin light chain variable region (IgV) by the gain of sIgM expression of the single cell sorted sIgM negative sub-clones of the Clone 18 and BACH2KO cell lines. The re-expression of BACH2 in BACH2KO cells (BACH2KO/BACH2^{R}) (III, Figure 2F) restored sIgM gain to the Clone 18 level (III, Figure 2B). The sIgM gain might be obtained by GCV and SHM events reversing the premature stop codon in *IgV* gene of the Clone 18 DT40 cells. The results demonstrate that the frequency of mutations in IgV is BACH2-dependent.

To reveal the specific spectrum of mutations, the rearranged *IgV* gene locus from Clone 18 and BACH2KO sub-clones was sequenced after 41 days of the sIgM gain assay. The sequencing revealed that BACH2KO cells had very low number of mutation events (as defined in *Materials and methods*) of both GCVs and SHMs, and no insertions and deletions were observed (III, Figure 2C and D). While Clone 18 sub-clones had one-third of analyzed sequences mutated, the BACH2KO sub-clones had only 8% with fewer mutations per DNA sequence (III, Figure 2C). Furthermore, the frequency of total mutation events, GCV, and SHM were more than 70% reduced in the absence of BACH2 (III, Figure 2E). Taken together, the results of the sIgM gain assay and sequencing of *IgV* gene loci clearly indicate that BACH2 is essential for the efficient generation of mutations in Ig locus. The data also demonstrate a new role for BACH2 in the regulation of GCV.

5.2.1 Disruption of BACH2 affects expression of genes with direct or indirect function in Ig diversification processes (III)

To identify the gene expression changes imposed by *BACH2* deletion, RNA sequencing (RNA-seq) from BACH2KO and Clone 18 cell line was performed. The RNA-seq analysis with Ingenuity Pathway Analysis uncovered that among 1355 genes differentially expressed (with an absolute fold change ≥ 1.4 and $p \leq 0.05$) in BACH2KO vs. Clone 18 cells (III, Figure 3C) there were genes with direct or indirect functions in Ig diversification processes such as V(D)J recombination, GCV, SHM and CSR (Table 7; III, Table S1). These genes encode transcription regulators *BATF*, *BCL6*, *ID2*, *NFKBIE, SMAD3* and NF B subunits (*REL* and *NFKB2*); enzymes *AICDA* (encoding AID), *RAG2*, *HMOX1*, *PARP3*, *POLH*, *POLL* and *REV3L*; and chaperones *DNAJA1* and *HSP90AB1* (role of these genes in the Ig diversification processes is summarized in Table 7).

Table 7. Differentially expressed genes in BACH2KO vs. Clone 18 cells with a function related to Ig diversification processes

Symbol	Entrez Gene Name	Fold change[a]	*p*-value[b]	Location[c]	Type(s)[c]	Function	References
AICDA	activation-induced cytidine deaminase	-1.495	0.001	Cytoplasm	enzyme	Initiates SHM, CSR, and GCV	(Arakawa et al., 2002a; Harris et al., 2002; Muramatsu et al., 2000; Revy et al., 2000)
BATF	basic leucine zipper ATF-like transcription factor	-1.82	0.002	Nucleus	transcription regulator	Directly controls AICDA expression	(Ise et al., 2011)
BCL6	B-cell CLL/lymphoma 6	-1.919	0.001	Nucleus	transcription regulator	Required for SHM and GCV	(Alinikula et al., 2011; Basso et al., 2012; Williams et al., 2016)
DNAJA1	DnaJ heat shock protein family (Hsp40) member A1	-1.902	<0.0001	Nucleus	other	Stabilizes cytoplasmic AID	(Orthwein et al., 2012)
EXOSC9	exosome component 9	1.414	0.0001	Nucleus	enzyme	RNA exosome targets AID to dsDNA	(Basu et al., 2011)
HMOX1	heme oxygenase 1	1.498	<0.0001	Cytoplasm	enzyme	HMOX1 product Fe^{2+} inhibits AID enzymatic activity	(Li et al., 2012a)
HSP90AB1	heat shock protein 90kDa alpha family class B member 1	-1.521	<0.0001	Cytoplasm	enzyme	Stabilizes cytoplasmic AID	(Orthwein et al., 2010)
ID2	inhibitor of DNA binding 2, HLH protein	-4.112	<0.0001	Nucleus	transcription regulator	Represses AICDA	(Gonda et al., 2003; Sayegh et al., 2003)
NFKBIE	NFKB inhibitor epsilon	-3.826	<0.0001	Nucleus	transcription regulator	Indirect regulation of AICDA through inhibition of NFKB	(Whiteside et al., 1997)
NFKB2	nuclear factor kappa B subunit 2	-1.511	0.002	Nucleus	transcription regulator	Regulates AICDA expression	(Park et al., 2009; Zan and Casali, 2013)
PARP3	poly(ADP-ribose) polymerase family member 3	-1.645	0.022	Nucleus	enzyme	Controls AID levels at switch regions (CSR)	(Robert et al., 2015)
PKIA	protein kinase (cAMP-dependent, catalytic) inhibitor alpha	3.383	0.001	Cytoplasm	other	Inhibits PKA	(Olsen and Uhler, 1991)
POLH	polymerase (DNA) eta	-2.506	<0.0001	Nucleus	enzyme	Involved in DNA repair, SHM	(Hirota et al., 2010; Kano et al., 2012; Li et al., 2013; Masuda et al., 2008)
POLL	polymerase (DNA) lambda	-2.125	<0.0001	Nucleus	enzyme	Base excision repair polymerase	(Braithwaite et al., 2005)
RAG2	recombination activating gene 2	-1.754	0.047	Nucleus	enzyme	V(D)J recombination	(Oettinger et al., 1990)
REV3L	REV3 like DNA-directed polymerase zeta catalytic subunit	-1.585	0.003	Nucleus	enzyme	Form the error-prone DNA polymerase zeta	(Gan et al., 2008; Okada et al., 2005)
REL	REL proto-oncogene, NF-kB subunit	-1.509	<0.0001	Nucleus	transcription regulator	Regulates AICDA expression	(Tran et al., 2010)
SMAD3	SMAD family member 3	-2.501	<0.0001	Nucleus	transcription regulator	Binds to AICDA regulatory region	(Tran et al., 2010)

[a] Threshold with absolute fold change >1.4
[b] $p<0.05$ considered as significant
[c] Classification of Location and Type(s) were derived with Ingenuity Pathway Analysis

Next, I compared the list of differentially expressed genes from the BACH2KO vs. Clone 18 cell line with available microarray data sets from murine *Bach2* knockout pre-B cells (Swaminathan et al., 2013) and activated B cells (Itoh-Nakadai et al., 2014). These analyses showed that the expression of *HMOX1*, *HPGDS*, *SLC9A9,* and *PARP3* was changed in a similar manner in all compared data sets, indicating that few BACH2 targets are independent of the B-cell developmental stage and are evolutionarily conserved between chicken and mouse.

5.2.2 The reduction of AID expression underlies the defect in mutation frequency in BACH2KO cells (III)

The disruption of *BACH2* decreased the expression of *AICDA* by 33% (Table 7 and III, Table S1). AICDA encodes the mutagenic enzyme AID that is critical for the initiation of SHM, CSR and GCV (Arakawa et al., 2002a; Harris et al., 2002; Muramatsu et al., 2000; Revy et al., 2000).

The Western blot analyses of whole cell lysates and subcellular fractionations showed that AID was reduced by 44.3% in whole cell lysates and by 57% in nuclear fractions of BACH2KO cells when compared with Clone 18 cells (III, Figure 4). Differences between mRNA, cytoplasmic and nuclear levels of AID in BACH2KO cells may result from post-transcriptional/translational regulations and active nuclear export of AID. The AID expression is controlled on multiple molecular levels that restrict expression of this mutagenic enzyme to activated B cells (GC and some extrafollicular B cells) (Cattoretti et al., 2006; Crouch et al., 2007; Muramatsu et al., 1999) and allows access to DNA only at specific time points of cell cycle (early G1 phase) (Wang et al., 2017). Indeed, in BACH2KO cells the expression of several genes encoding proteins relevant for AID stability and activity was changed e.g. *PKIA*, *DNAJA1,* and *HSP90AB* (Table 7; III, Figure S2, Table S2). PKIA is an inhibitor of protein kinase A (PKA) which increases the activity of AID by phosphorylating the serine 38 (Basu et al., 2005; Basu et al., 2009; Cheng et al., 2009; McBride et al., 2008; Pasqualucci et al., 2006). Therefore, over 3-fold increased expression of *PKIA* in BACH2KO cells may contribute to lower AID activity. Furthermore, reduced expression of molecular chaperones *DNAJA1* and *HSP90AB1*, known to stabilize cytoplasmic AID (Orthwein et al., 2010; Orthwein et al., 2012), could explain the difference between mRNA and total protein levels of AID observed in BACH2KO cells; especially since *DNAJA1* inactivation impairs AID stability leading to the loss of AID biological activity (Orthwein et al., 2012).

Importantly, AID expression in BACH2KO/BACH2^{R} cells was restored even above Clone 18 level (III, Figure 4A), at the same level as a *BACH2* expression

in BACH2KO/BACH2^{R} cells (III, Figure 2F), indicating that BACH2 positively regulates AID expression. BACH2 has been shown to act as an activator in addition to being a transcriptional repressor (Igarashi et al., 2017; Oyake et al., 1996). Thus, the question arises whether BACH2 upregulates *AICDA* expression directly or indirectly.

The complementation of AID expression in BACH2KO cells restored the mutation frequency to the levels observed in Clone 18 cells (III, Figure 5). This experiment showed that the reduction of SHM and GCV frequency in BACH2KO cells is AID-dependent and can be overcome by enhanced AID expression.

5.2.3 BACH2 regulates AID expression in BLIMP1-independent manner (III)

In murine *Bach2* knockout B cells, the reduction of AID expression and thus impairment of SHM and CSR was accompanied by a rapid increase of *Prdm1* expression upon LPS stimulation (Muto et al., 2004). However, deletion of both *Prdm1* and *Bach2* in mouse restored the *Aicda* expression and CSR (Muto et al., 2010), suggesting that elevated expression of *Prdm1* caused reduction of *Aicda* expression. In fact, BLIMP1 (encoded by *PRDM1*) was found to directly repress *AICDA* (Minnich et al., 2016).

To verify whether inactivation of *BACH2* in DT40 B cells caused de-repression of *PRDM1* and thus reduced AID levels, the expression of *PRDM1* was checked using RNA-seq and RT-qPCR of Clone 18, BACH2KO and *PRDM1* expressing *BCL6* knockout (Alinikula et al., 2011) cells. The BCL6 knockout cells acted as a positive control for significant *PRDM1* expression. The *PRDM1* expression was hardly detectable in Clone 18 DT40 cell line both in RNA-seq and RT-qPCR as expected since this cell line is not a plasmacytic cell line. The *PRDM1* was unchanged in BACH2KO cells (III, Figure 3B). No changes in the expression of BLIMP1 target genes were observed [BLIMP1 target genes in accordance with (Minnich et al., 2016)] such as *IRF4*, *SKIL*, *PAX5*, *GFI1*, *CNST*, *WNT10A*, *MCOLN2*, *TRAM2*, *SSR2*, *DERL1*, *IL6ST* and *MZB1* in BACH2KO vs. Clone 18 cells (with an absolute fold change ≥ 1.4 and $p \leq 0.05$) (III, Table S1). This strongly speaks against a biological significance of expression of *PRDM1* in BACH2KO cells. Hence, I conclude that BACH2 regulates AID expression in a BLIMP1-independent manner in DT40 B cells.

These findings differ from those from the *Bach2* knockout mouse model in the regulation of *AICDA* expression. Reasons for this difference might be that DT40 cells continuously express AID and perform GCV/SHM whereas murine B cells require stimulation for activation and upregulation of AID expression. The LPS

stimulation of mouse B cells in the absence of *Bach2* leads to the de-repression of BLIMP1 and thus downregulation of AID expression (Muto et al., 2010; Muto et al., 2004). The DT40 B cells are unresponsive to the LPS stimulation due to the lack of *TLR4* and *CD14* expression (average RPKM values from RNA-seq in Clone 18 cells are 0.03 and 0.02, respectively) that mediate LPS stimulation. Therefore LPS had no effect on *PRDM1* expression (data not shown). The data clearly demonstrate that AID expression is BACH2-dependent and BLIMP1-independent.

Inactivation of *BACH2* reduced the expression of several additional transcription factors such as BCL6, BATF, and NF- B known to regulate *AICDA* expression (Alinikula et al., 2011; Ise et al., 2011; Basso et al., 2012; Tran et al., 2010). While the restoration of AID expression and sIgM gain were observed (III, Figure 1B, and 4A) upon BACH2 reintroduction, the expression of neither *BCL6* nor *BATF* was restored in BACH2KO/BACH2^{R} cells. These results suggest that BCL6 and BATF do not cause the reduced AID expression. This would support the idea that BACH2 regulates *AICDA* directly.

The promoter region of chicken *AICDA* (chicken genome version galGal4) has a BACH2 binding motif (TGACTCA) corresponding to the AP-1-like sequence to which BACH2 can bind at high concentrations (Oyake et al., 1996). To check BACH2 binding to *AICDA* promoter, I tested several commercially available and even custom made anti-BACH2 antibodies, but none of them recognized chicken BACH2. Moreover, I created an HA-tagged BACH2 to be able to immunoprecipitate it with anti-HA tag antibodies, but the crosslinking destroyed the epitope precluding BACH2 immunoprecipitation and thus validation of BACH2 binding to the chicken *AICDA* promoter. However, the ability of BACH2 binding to the *AICDA* promoter and enhancer regions is observed in the BACH2 ChIP-seq experiments performed in a human lymphoma cell line (OCI-Ly7) (Huang et al., 2014) and mouse regulatory T cells (Roychoudhuri et al., 2013). Thus, BACH2 may even regulate AID expression directly. All these results show clearly that BACH2 has a novel function in controlling GCV and SHM in DT40 B cells.

6 CONCLUSIONS

The role of IRF4 and BACH2 in B cell development has been extensively investigated in recent years and application of genome-wide analysis methods such as ChIP-seq, RNA-seq and microarray analyses allowed identification of more than hundreds of potential IRF4 and BACH2 target genes. However, only a few of these direct targets have been thoroughly studied in a limited number of model systems, leaving an open space for further research to better understand the complex networks of transcription factors behind the differentiation of B cells into high-affinity antibody-secreting plasma cells.

This thesis aimed at investigating the role of IRF4 in B cells using an IRF4-deficient cell line. Studies conducted in this cell line in combination with genetic complementation of IRF4 found new IRF4 direct target genes such as *IRF8* and *SHIP*, demonstrating for the first time the important role of IRF4 in the regulation of BCR signaling. IRF4 directly upregulates *SHIP* and downregulates *IRF8* expression that results in a lowered BCR signaling threshold and in unbalanced signal transduction to the major signaling pathways such as ERK and PI3K/AKT (I). These findings shed light on the germinal center response where antigen-activated B cells lack *IRF4* expression and have enhanced expression of *IRF8*, similarly to the *IRF4* knockout DT40 B cells.

The second goal of this thesis, the elucidation of a role for IRF4 in antibody secretion, was reached by the generation of the $IRF4^{-/-}/BCL6^{-/-}$ double knockout cell line and overexpression of IRF4 and BLIMP1 in this cell line. The results indicated that IgM secretion can be induced by ectopic overexpression of BLIMP1 only in the presence of IRF4, or by the overexpression of IRF4 when BLIMP1 is de-repressed in the absence of *BCL6*. Hence BLIMP1 is insufficient to induce antibody secretion in the absence of IRF4 (II). However, further studies are needed to reveal the exact mechanism of IRF4-mediated induction of antibody secretion.

This work also has provided a clear insight into the role of BACH2 in AID-mediated immunoglobulin gene diversification that produces high-affinity antibodies. BACH2 deficiency in DT40 cells resulted in the BLIMP1-independent reduction of expression and nuclear pool of AID resulting in lowered frequency of immunoglobulin gene conversion and somatic hypermutation (III). These findings are novel since a role of BACH2 in gene conversion has not been investigated earlier. The previous studies conducted in mouse models suggested that BACH2 prevents plasmacytic gene expression program and BLIMP1 expression allowing AID activity and somatic hypermutation (Muto et al., 2010).

In conclusion, this thesis expands the knowledge of transcriptional network regulating B cell development and function by revealing a role for IRF4 in BCR signaling, a function in antibody secretion, and a different perspective on the BACH2 function in immunoglobulin diversification processes.

All these results are important for understanding the basic transcriptional control of B cells and B cell differentiation process into memory B cells and antibody-producing plasma cells. High-affinity pathogen-neutralizing antibodies are important in controlling infections and are sought in vaccinations. Furthermore, the role of IRF4 and BACH2 in B cell development, their role in lymphoid malignancies (Basso and Dalla-Favera, 2015; Shukla and Lu, 2014) and their possible role in autoimmune diseases (Afzali et al., 2017; Igarashi et al., 2017; Xu et al., 2012) are medically relevant to study further.

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