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REGULATION OF B CELL GENE EXPRESSION AND FUNCTION BY IKAROS, HELIOS AND BCL6

by

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To my family

ABSTRACT

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B lymphocytes constitute a key branch of adaptive immunity by providing specificity to recognize a vast variety of antigens by B cell antigen receptors (BCR) and secreted antibodies. Antigen recognition activates the cells and can produce antibody secreting plasma cells via germinal center reaction that leads to the maturation of antigen recognition affinity and switching of antibody effector class. The specificity of antigen recognition is achieved through a multistep developmental pathway that is organized by interplay of transcription factors and signals through BCR.

Lymphoid malignancies arise from different stages of development in abnormal function of transcriptional regulation. To understand the B cell development and the function of B cells, a thorough understanding of the regulation of gene expression is important. The transcription factors of the Ikaros family and Bcl6 are frequently associated with lymphoma generation. The aim of this study was to reveal the targets of Ikaros, Helios and Bcl6 mediated gene regulation and to find out the function of Ikaros and Helios in B cells.

This study uses gene targeted DT40 B cell lines and establishes a role for Ikaros family factors Ikaros and Helios in the regulation of BCR signaling that is important at developmental checkpoints, for cell survival and in activation. Ikaros and Helios had opposing roles in the regulation of BCR signals. Ikaros was found to directly repress the *SHIP* gene that encodes a signaling lipid-metabolizing enzyme, whereas Helios had activating effect on *SHIP* expression. The findings demonstrate a balancing function for these two Ikaros family transcription factors in the regulation of BCR signaling as well as in the regulation of gene expression. Bcl6 was found to repress plasma cell gene expression program while maintaining gene expression profile of B cells. Analysis of direct Bcl6 target genes suggested novel mechanisms for Bcl6-mediated suppression of plasma cell differentiation and promoting germinal center phenotype.

Key words: Ikaros, Helios, Bcl6, regulation of transcription, B cell, plasma cell

TIIVISTELMÄ

Jukka Alinikula
Ikaros, Helios ja Bcl6 B-solujen geeniluennan ja toiminnan säätelijöinä
Lääketieteellinen Mikrobiologia ja Immunologia
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B-lymfosyytit edustavat hankitun immuniteetin haaraa, jonka tehtävänä on tunnistaa suuri määrä eri antigeenejä antigeenireseptorillaan ja tuottaa monimuotoisia vasta-aineita verenkiertoon. Eri antigeenien tunnistus aktivoi B-solut tuottamaan plasmasoluja itukeskusreaktion kautta. Tämä parantaa vasta-aineen affiniteettia ja johtaa immunoglobuliinin luokanvaihtoon. Antigeenin tunnistuksen tarkkuus saavutetaan B-solukehityksen aikana geeniluennan säätelyn ja antigeenireseptorin signaloinnin avulla.

B-solujen maligniteetit syntyvät eri kehitysvaiheissa häiriintyneen geenisäätelyn seurauksena. B-solujen kehityksen toiminnan ymmärtämiseksi tarvitaan tarkempaa tietoa geenisäätelystä. Bcl6 ja Ikaros-perheen geenisäätelytekijöiden poikkeavat toiminnat ovat yhteydessä lymfoomien syntyyn. Tämän tutkimuksen tarkoituksena oli löytää Ikaroksen, Helioksen ja Bcl6:n kohdegeenit ja selvittää Ikaroksen ja Helioksen toimintaa B-soluissa.

Tutkimuksessa käytettiin poistogeenisiä DT40 B-solulinjoja, joilla saadut tulokset osoittavat Ikaroksen ja Helioksen säätelevän B-solureseptorin viestintää, joka on tärkeä säätelijä B-solukehityksen eri tarkastuspisteissä, joissa ratkaistaan solujen henkiin jääminen ja aktivaaktio. Ikaroksella ja Helioksella oli päinvastainen rooli B-solureseptorin viestinnässä. Ikaros estää suoraan *SHIP*-geenin luentaa. SHIP säätelee solukalvon rasvamolekyylien välittämää viestintää. Helios päinvastoin lisää kyseisen geenin luentaa. Nämä tulokset osoittavat Ikaroksen ja Helioksen tasapainottavan toisiaan B-solureseptoriviestinnnän ja geeniluennan säätelyssä. Bcl6 estää plasmasolugeenejä ja ylläpitää itukeskusgeenien ilmentymistä. Suorien Bcl6-kohdegeenien analyysi paljasti uuden mekanismin, jolla Bcl6 estää plasmasolujen erilaistumista ja ylläpitää itukeskukseen liittyviä ominaisuuksia.

Avainsanat: Ikaros, Helios, Bcl6, geeniluennan säätely, B-solu, plasmasolu

CONTENTS

ABBREVIATIONS

Ab	antibody
Ag	antigen
AID	activation-induced cytidine deaminase
ARF	ADP ribosylation factor
ATM	ataxia telangiectasia mutated
Bach2	BTB and CNC homology 2
BAFF	B cell activating factor
Bcl6	B cell lymphoma 6
BCoR	Bcl6 corepressor
BCR	B cell receptor
BER	base-excision repair
Blimp-1	B lymphocyte-induced maturation protein
BLNK	B cell linker protein
BTB/POZ	bric à brac, tramtrack, broad-complex/pox virus and zinc finger
BTK	Bruton's tyrosine kinase
Cbl	Casitas B-lineage lymphoma
CD	cluster of differentiation
CHEK1	checkpoint kinase 1 homolog
ChIP	chromatin immunoprecipitation
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CSR	class-switch recombination
CtBP	C-terminal binding protein
DAG	diacylglycerol
DTT	dithiothreitol
EBI2	Epstein-Barr virus induced gene 2
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ETP	early thymic progenitor
FDC	follicular dendritic cell
Flt3	fms-related tyrosine kinase 3
GC	germinal center
GEO	Gene Expression Omnibus
HSC	hematopoietic stem cell
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
IL-7	interleukin-7
IL-7Rα	interleukin-7 receptor, alpha chain
INPP5D	inositol polyphosphate-5-phosphatase D
IP_3	inostiol-1,4,5-trisphosphate
IRE1	inositol-requiring enzyme 1
IRF	interferon regulatory factor

ITAM	immunoreceptor tyrosine-based activation motif
LMPP	lymphoid-primed multipotent progenitor
LSK	$Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$
mAb	monoclonal antibody
MITF	microphthalmia-associated transcription factor
NCoR	nuclear receptor co-repressor 1
NF-κB	nuclear factor kappa B
NHEJ	non-homologous end joining
NK	natural killer
Pax5	paired box protein 5
PH	pleckstrin homology
$PI(3,4)P_2$	phosphatidylinositol-3,4-bisphosphate
$PI(3,4,5)P_3$	phosphatidylinositol-3,4,5-trisphosphate
$PI(4,5)P_2$	phosphatidylinositol-4,5-bisphosphate
PLCγ2	phospholipase Cγ-2
Prdm1	positive regulatory domain containing 1
Rag	recombination activating gene
SDP-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	src-homology 2
SHIP	SH2-containing inositol phosphatase
SHM	somatic hypermutation
sIgM	surface immunoglobulin M
Ska3	spindle and kinetochore associated complex subunit 3
SLC	surrogate light chain
SMRT	silencing mediator of retinoid acid and thyroid hormone receptor
Stat	signal transducer and activator of transcription
T_{FH}	follicular T helper cell
TSS	transcription start site
UNG	uracil-DNA glycosylase
URE	upstream regulatory element
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-IV):

I Nera K-P, Alinikula J, Terho P, Narvi E, Törnquist K, Kurosaki T, Buerstedde JM, Lassila O. (2006). Ikaros has a crucial role in regulation of B cell receptor signaling. *Eur J Immunol*. 36:516-525

II Alinikula J, Kohonen P, Nera K-P, Lassila O. (2010). Concerted action of Helios and Ikaros controls the expression of inositol 5-phosphatase SHIP. *Eur J Immunol.* 40:2599-2607

III Alinikula J, Nera K-P, Lassila O. (2010). Bcl6 directly regulates Bach2 and UNG expression in B cell to plasma cell differentiation. *Submitted*

IV Alinikula J, Lassila O, Nera K-P. (2006). DT40 mutants: a model to study transcriptional regulation of B cell development and function. *Subcell Biochem.* 40:189-205

1 INTRODUCTION

Adaptive immune system faces a challenge of recognizing countless potentially harmful foreign agents while avoiding to react against self structures. B lymphocytes represent a major branch of this system. They provide molecular specificity for the recognition and clearance of pathogens by producing a vast repertoire of immunoglobulins. Cells of the B lineage extend their genomic information by DNA rearrangements and mutations to generate various specificities during a multistep development. The immunoglobulin molecule is expressed on the cell surface as a major component of B cell antigen receptor and secreted as antibodies.

Antigen encounteri by a mature B cell gives an activating signal via the B cell receptor and leads eventually to terminal differentiation into a plasma cell, an antibody-secreting factory. The developing B cell deals with functions such as prevention of autoreactivity, rapid proliferation and active mutation of the genome, all potentially dangerous for the organism if not properly controlled. Autoimmune diseases and lymphoid malignancies are known to arise from different stages of B cell development in the case of malfunction of their regulatory system.

The differentiation process is guided by transcription factors that regulate the expression of appropriate genes. A thorough investigation of functions and target genes of these factors is fundamental in the effort to understand how B cells work and how they are kept under control.

Ikaros family transcription factors function in the generation of lymphoid system, and Ikaros is needed for the development of B cells. In this study, the function of Ikaros family members Ikarors and Helios are investigated, with special emphasis on the founding member Ikaros. Since Ikaros-deficient mice have a block early in lymphoid development pathway and do not have B cells, the mouse model has revealed little information on the function of Ikaros in B cells and is therefore not thoroughly understood. The expression of transcription factor Bcl6 (B cell lymphoma 6) is more restricted to germinal centers, the site for somatic hypermutation, immunoglobulin class switching and induction of memory and plasma cell differentiation. Bcl6 is essential for germinal center formation and function of germinal center B cells. The network of transcription factors guiding the subsequent differentiation to plasma cells and direct target genes of Bcl6 are not completely understood. In this thesis, the function of Bcl6 in the network is analyzed by finding target genes of Bcl6.

2 REVIEW OF THE LITERATURE

2.1 Shaping up the B cell lineage

2.1.1 Early hematopoiesis

Hematopoietic stem cells (HSC) of the adult bone marrow give rise to all blood cells. As the stem cells develop, they start progressive specification (priming of the cell fate or induction of lineage-specific gene expression program) and commitment (repression of alternative gene expression programs) into certain cell types (Figure 1). The hierarchy and relationship of the lineages is a matter of continuous controversy and several models have been proposed (Katsura, 2002; Lai & Kondo, 2006; Ceredig et al., 2009; Kawamoto & Katsura, 2009; Yoshida et al., 2010). HSCs with long-term (CD34) and short-term ($CD34^+$) self-renewing potential as well as most of the multipotent progenitors (MPPs), that have lost self-renewing potential, can be purified from the mouse bone marrow by being positive for expression of Sca1 and c-Kit (CD117), but lacking high levels of classical lineage markers (Lin^-). This population is thereby called LSK ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$) and is very heterogeneous (reviewed by Ye & Graf, 2007). The identification of LMPPs (lympho-myeloid restricted or lymphoid primed MPPs) that can produce granulocytes, macrophages, B, T and NK cells, but have a very poor capacity to produce megakaryocytes or erythrocytes (Adolfsson et al., 2005) supports a scheme that myeloid cells do not necessarily have a common precursor (Lai & Kondo, 2006; Yoshida et al., 2006). Also contrasting evidence exists (Akashi et al., 2000).

The hierarchical tree depiction of hematopoiesis was formulated on the basis of findings that thymic precursor cells give rise to B, T and natural killer (NK) cells (Wu et al., 1991; Matsuzaki et al., 1993; Kondo et al., 1997) and are therefore termed common lymphoid progenitor (CLP) cells. Later, these findings have received support from several experiments. CLP was defined as a progenitor pool that has no lineage specific cell surface molecules but expression of interleukin-7 receptor and low expression of c-kit ($IL\text{-}7R^+c\text{-}kit^{low}$) (Kondo et al., 1997). Later also the existence of common myeloid progenitors (CMPs) was demonstrated (Akashi et al., 2000). Recently, the originally described CLP was reported to consist of functionally distinct subsets, of which the Flt3 (Flk2, CD135) expressing cells have strong *in vivo* and *in vitro* potential to generate B, T, NK and DC cells but not myeloid cells (Karsunky et al., 2008; Serwold et al., 2009) strongly supporting the existence of CLP.

However, introduction of methods using clonal analysis of hematopoietic precursors have lead to accumulation of evidence challenging the existence of CLP (Bell & Bhandoola, 2008; Wada et al., 2008). The new evidence suggests that immune cell progenitors retain significant myeloid potential supporting a myeloid-based model for hematopoiesis (Kawamoto & Katsura, 2009).

As recent findings increasingly reveal plasticity of lineages, the classification based on tree-depiction may be too simplified, and different views, such as the one based on pairwise relationship, may prove to be more useful (Ceredig et al., 2009). This model has closely related lineages placed next to each other and is supported by transcription factor expression and function in neighboring cell lineages. The potential of a cell to generate different cell types at any given time is dictated by specific

transcription factors and other regulators such as micro-RNAs. Finding out the functions of these transcription factors *per se* will be useful in understanding of the differentiation of blood lineages.

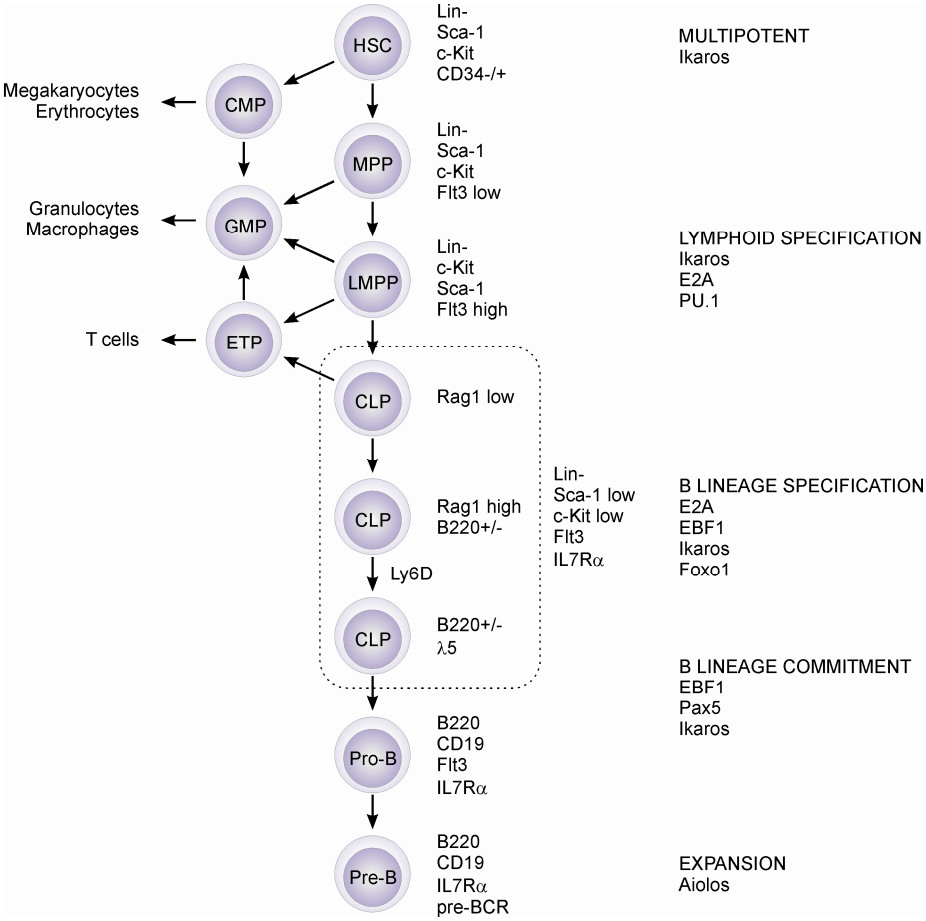


Figure 1 | A schematic presentation of lineage restriction to B cell fate in the bone marrow
The hematopoietic stem cell (HSC) gives rise to all the blood cells. According to the current view megakaryocyte-erythrocyte pathway diverges earliest into separate lineage, whereas myeloid lineages and lymphoid lineages proceed in a common pathway. Expression of proteins that are indicative of the developmental stage are indicated immediately right to the corresponding cell. Progenitor cell that do not express high levels of any classical lineage markers (Lin-) are indicated. The subcategorization of CLP population is according to Mansson et al., 2010. CMP is indicated despite its role in giving rise to all myeloid cell types is controversial. GMP, Granulocyte/macrophage progenitor; MPP, multipotent progenitor; CMP, common myeloid progenitor; ETP, early thymic progenitor; CLP, common lymphoid progenitor.

2.1.2 Specification to lymphoid lineages

The hallmarks of lymphoid lineage priming (lymphoid specification or induction of lymphoid lineage-specific gene expression program) in the bone marrow are the gradual upregulation of Flt3, IL-7Rα, Rag1 and Rag2 (Figure 1, Adolfsson et al., 2005; Lai & Kondo, 2006). Subcategorization of hematopoietic stem cells according to

expression of Flt3 (fms-like tyrosine kinase 3) has revealed the existence of lymphoid primed multipotent progenitors (LMPPs), that can generate lymphoid (CLP) and myeloid (GMP) progeny but not erythro-megakaryocyte progeny (Adolfsson et al., 2005). Mice with defects in the Flt3 signaling system have impaired development of CLPs (Sitnicka et al., 2002). Even more profound effect is observed in double-mutant *flt3*$^{-/-}$*Il7ra*$^{-/-}$ mice that entirely lack B-lymphocytes in the bone marrow (Vosshenrich et al., 2003). IL-7 provides survival signals to developing B cells and allows B cell development of the CLPs (Miller et al., 2002), and IL-7Rα (encoded by *Il7r* gene) is expressed from CLP stage to pre-B cell stage (Figure 1). In priming of the lymphoid lineages the transcription factors PU.1, Ikaros and E2A have prominent roles (Figure 1 and 2).

PU.1 is an ETS-family transcription factor that is expressed in myeloid and lymphoid cells (Klemsz et al., 1990; Hromas et al., 1993). Mice with homozygous inactivation of *Spfi1* (the gene encoding PU.1) die around birth and lack B, T, monocytic and granulocytic cells, while erythrocytes and megakaryocytes develop normally in fetal liver (Scott et al., 1994; McKercher et al., 1996). Lymphoid-primed multipotent progenitor (LMPP) compartment ($Flt3^{+}Lin^{-}AA4.1^{+}$) in *Spfi*$^{-/-}$ fetal liver is reduced and cannot produce B cell precursors (Scott et al., 1997). Since elimination of PU.1 in adult mice leads to a lack of identifiable CLPs and to a loss of lymphoid lineages, PU.1 seems to function before or at the level of CLP stage (Dakic et al., 2005).

It has been suggested that PU.1 is needed in a dose-dependent manner to regulate the development between B lymphocyte and macrophage fates, as ectopic expression of PU.1 in fetal liver of *Sfpi1*$^{-/-}$ mice produces macrophages with high PU.1 expression and B cells with low PU.1 expression (DeKoter & Singh, 2000). These expression levels correlate with the normal PU.1 expression in B cells and macrophages (Nutt et al., 2005). The evidence that low level of PU.1 expression is required for B cell development comes from the findings that the B cell development is impaired in *Sfpi1*$^{-/-}$ mice (Scott et al., 1994; McKercher et al., 1996; Scott et al., 1997) and knockdown of PU.1 in hematopoietic progenitors induces B lineage development (Zou et al., 2005). In contrast to this idea, genetic perturbations of an upstream regulatory element (URE) or the first two start codons of *Spfi1*, which reduce the expression of PU.1, inhibit early development of conventional B2 cells (Rosenbauer et al., 2006; Houston et al., 2007). Instead of regulating B versus macrophage lineage decision, PU.1 seems to specify or maintain the innate-like B1 fate at the expense of B2 fate. The deletion of URE, that functions as an enhancer in B cells, increases B1 B cell development while ablating B2 cell development (Rosenbauer et al., 2006). Similarly, CD19-Cre-mediated inactivation of PU.1 in developing B lineage cells results in a shift from B2 to B1-like cells (Ye et al., 2005). Importantly, conditional deletion of PU.1 in the committed B cells and *in vitro* inactivation in CLPs allow relatively normal B cell development and function (Iwasaki et al., 2005; Polli et al., 2005; Ye et al., 2005). Thus, these studies collectively suggest that PU.1 is needed to specify lymphoid progenitors but is not critically needed for later B cell development.

PU.1 regulates the lymphoid priming in part by regulating the expression of the alpha chain of interleukin-7 receptor (IL-7Rα). PU.1-deficient cells lack IL-7Rα expression, PU.1 binds to *Il7r* promoter and re-expression of IL-7Rα in *Sfp1*$^{-/-}$

progenitor cells rescues the development of B cell compartment (DeKoter et al., 2002). The CLPs of IL-7 receptor-deficient mice are severely compromised in their ability to differentiate even to the earliest pro-B cell stage in the adult bone marrow (Carvalho et al., 2001; Miller et al., 2002). PU.1 also regulates the expression of Flt3 (Carotta et al., 2010) that is required for efficient formation of the CLP and subsequent development of pro-B and pre-B cells (Mackarehtschian et al., 1995).

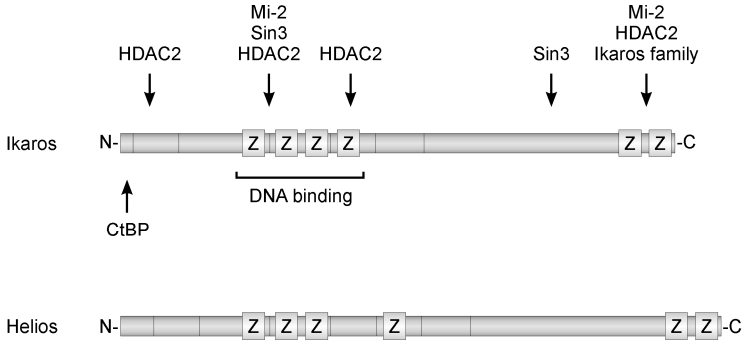


Figure 2 | Schematic presentation of domain and exon organization of Ikaros and Helios
The N-terminal DNA binding domain consists of four zinc fingers (Z) that can be alternatively spliced to produce several isoforms with zero to four zinc fingers resulting in proteins with varying DNA binding capability. The C-terminal zinc finger domain mediates homomerization and heteromerization with other Ikaros family members. Ikaros interacts with corepressor complexes through regions identified with arrows and the interactions are conserved between family members (Koipally & Georgopoulos, 2000, 2002). The exon-intron organizations are depicted according to *Gallus gallus* proteins.

Ikaros, the founding member of the Ikaros family, contributes to multiple aspects of hematopoietic development and is essential for normal lymphocyte development and homeostasis (Georgopoulos et al., 1992; Georgopoulos et al., 1994; Hahm et al., 1994; Winandy et al., 1995; Wang et al., 1996; Ng et al., 2009; Papathanasiou et al., 2009). The *Ikaros* gene is expressed as multiple isoforms. The full-length Ikaros protein has an N-terminal zinc finger domain containing four zinc fingers that mediate DNA-binding and a C-terminal zinc finger domain that mediates self-dimerization/multimerization or heteromerization with other family members such as Helios an Aiolos (Figure 2). The isoforms that lack the functional DNA-binding domain, such as Ik-6, can act as dominant negative isoforms as they retain the functional dimerization domain (Winandy et al., 1995; Sun et al., 1996). These isoforms occur naturally but their expression is abnormally high in leukemic cells (Sun et al., 1999a; Sun et al., 1999b; Sun et al., 1999c). The individual functions of the other isoforms are not known. The *Ikaros* gene is expressed in all hematopoietic lineages including hematopoietic stem cells and multipotent progenitors (Morgan et al., 1997; Kelley et al., 1998; Klug et al., 1998; Papathanasiou et al., 2009). Mice with loss-of-function and dominant negative mutations in *Ikaros* gene have severely impaired capacity to produce lymphoid lineages (Georgopoulos et al., 1994; Wang et al., 1996; Ng et al., 2009). Ikaros-null mice lack all B, NK and fetal T cells, demonstrating a persistent block in B and NK cell development. Some T cell progenitors in thymus and mature T cells in the periphery are found (Wang et al., 1996). The myeloid differentiation remains relatively normal in Ikaros-deficient cells as granulocytes and

macrophages as well as their progenitors are present in normal to increased numbers (Nichogiannopoulou et al., 1999; Yoshida et al., 2006). Mice with hypomorphic Ikaros mutation, that reduces but not fully prevents the expression of Ikaros, fail to undergo pro-B to pre-B cell transition and their bone marrow cells do not form colonies in response to IL-7 *in vitro* (Kirstetter et al., 2002). In line with these findings Ikaros seems to regulate the expression of several genes promoting lymphoid development, such as *TdT*, *Rag1*, *Rag2, λ5*, *Flt3* and *Il7r* (Figure 3, Kirstetter et al., 2002; Yoshida et al., 2006).

Ikaros-deficient progenitors lack Flt3 expression (Nichogiannopoulou et al., 1999) and Flt3 expression is highest in hematopoietic progenitors that express Ikaros (Yoshida et al., 2006) suggesting that Ikaros regulates Flt3 expression and therefore the apparent entry into LMPP stage. However, *Ikaros*-promoter driven reporter gene studies have revealed that Ikaros-deficient mice are not deficient in LMPP (Yoshida et al., 2006). These LMPPs that do not express Ikaros or Flt3 can differentiate into myeloid but not lymphoid pathways (Yoshida et al., 2006), suggesting that lymphoid specification takes place at or after the LMPP stage. As the effect of Ikaros null mutation affects more profoundly B lineage than T lineage development (Wang et al., 1996; Yoshida et al., 2006), Ikaros may have a prominent role in directing the development into CLP stage that gives rise to B cell precursors (Bryder & Sigvardsson, 2010). Mechanistically, this may be due to reduction of both IL-7Rα and Flt3 expression, as *Flt3*$^{-/-}$*Il7r*$^{-/-}$ double deficient mice have more profound phenotype than deletion of either of these genes alone and lack B lineage development in the bone marrow (Mackarehtschian et al., 1995; Vosshenrich et al., 2003). This also suggests overlapping functions of Ikaros and PU.1 in priming of the lymphoid fate.

E2A is a basic helix-loop-helix transcription factor that is also needed for lymphoid priming in multipotent progenitors (Dias et al., 2008; Semerad et al., 2009). E2A is expressed in the earliest lymphoid progenitors and is required for the B cell development, as E2A-deficient mice have a developmental block at the pro-B cell stage (Bain et al., 1994; Zhuang et al., 1994; Borghesi et al., 2005), the earliest defined progenitors in the B cell lineage (Figure 1 and 4). E2A occurs as two proteins, E12 and E47, (encoded by *Tcfe2a*) that arise through differential splicing of the transcript (Murre et al., 1989). In the absence of both E2A proteins the number of LMPPs and CLPs is severely reduced (Dias et al., 2008; Semerad et al., 2009).

Furthermore, E2A is necessary to maintain EBF1 expression and thereby also for specification to B lineage (Figure 3) synergistically with Ikaros, PU.1 and Gfi1 (Kwon et al., 2008). The E2A-deficient progenitor cells do not express EBF1 and Pax5 properly, but re-expression of EBF1 or Pax5 in these cells circumvents the developmental block (Bain et al., 1994; Seet et al., 2004; Kwon et al., 2008), suggesting a main role of E2A in priming the lymphoid development. Thus, Ikaros, PU.1 and E2A together prime the hematopoietic progenitors to lymphoid lineages and allow subsequent specification to the B cell lineage.

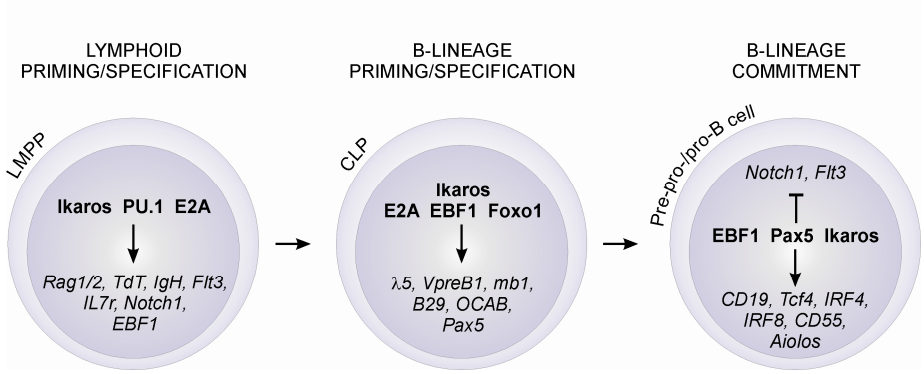


Figure 3 | Gene regulatory programs in shaping the B lineage
Key transcription factors shape the gene expression profile required for stepwise lineage development. The previous steps induce the expression of the transcription factors that set up the expression in the next stage. After the B-lineage commitment phase the development is pre-BCR and BCR-dependent. LMPP, lymphoid-primed multipotent progenitor; CLP, common lymphoid progenitor.

2.1.3 Specification to B lineage

The requirement of E2A also for the priming to the B cell fate can at least partly be accounted for promoting of EBF1 expression (Lin et al., 2010). EBF1 is essential for the development of functional B cells (Lin & Grosschedl, 1995). It is first expressed in CLPs and functions in a network, together with E2A, Pax5 and Foxo1, regulating many genes involved in early B cell development, such as *mb-1*, *B29*, *Vpre-B1* and *Pax5* (Hagman et al., 1993; O'Riordan & Grosschedl, 1999; Sigvardsson et al., 2002; Medina et al., 2004; Lin et al., 2010). EBF1 is dispensable for the generation of CLPs, but is crucial for the specification and progression of the B cell program. Inactivation of EBF1 results into an early block in B cell development at the stage of a lymphoid progenitors that express Flt3, IL-7R and B220 (Medina et al., 2004) and in the failure to express B cell genes such as *mb-1*, *B29*, *λ5* and *VpreB1* (Lin & Grosschedl, 1995). Importantly, ectopic expression of EBF1 in mice deficient of E2A, IL-7, IL-7Rα, Ikaros or PU.1 is sufficient to overcome the developmental arrests in these mice (Medina et al., 2004; Seet et al., 2004; Dias et al., 2005; Kikuchi et al., 2005; Reynaud et al., 2008) suggesting that EBF1 acts downstream of these effectors.

The expression of *EBF1* is regulated by two different promoters that drive the expression of two different EBF1 proteins, EBF1α and EBF1β, that differ in their first 14 amino acids (Roessler et al., 2007). The activity of the distal promoter of EBF1α is regulated by IL-7 signaling, E2A and by EBF1 autoregulation, whereas the proximal promoter of EBF1β is controlled by Pax5, Ets1 and PU.1 (Smith et al., 2002; Roessler et al., 2007; Kikuchi et al., 2008). Expression and binding site analyses suggest that EBF1 regulates directly the expression of transcription factors Pax5, Pou2af1 (OcaB), and Foxo1 (Zandi et al., 2008; Lin et al., 2010) that are also themselves needed for B cell development (Kim et al., 1996; Nielsen et al., 1996; Schubart et al., 1996; Brunner et al., 2003; Hu et al., 2006). Pou2af1 has been reported to regulate the expression of the Ets transcription factor SpiB (Bartholdy et al., 2006) as well as genes encoding proteins involved in signaling and cell cycle regulation (Kim et al., 2003).

2.1.4 Commitment to B lineage

B cell commitment takes place before or concurrently with the onset of CD19 expression on the cell surface at the pro-B cell stage (Figure 1). Lymphoid restricted progenitors have significant plasticity before this stage (Rumfelt et al., 2006; Mansson et al., 2010). Pre-pro-B cell fraction of bone marrow cells ($B220^+CD19^-Flt3^+IL\text{-}7R^+$) contains approximately 50% B-lineage committed cells, while the remaining cells retain potential for both B and T cell lineages (Mansson et al., 2010). The signaling components of the B cell antigen receptor (BCR) or pre-BCR, Igα (encoded by *mb-1*) and Igβ (encoded by *B29*), are first expressed on pro-B cells in complex with calnexin (Nagata et al., 1997). The expression of Igα/Igβ continues throughout B cell development.

Expression of Pax5 marks the commitment to B lineage and is essential for maintenance of B lineage program as Pax5-deficient cells display great developmental plasticity (Nutt et al., 1999; Rolink et al., 1999b; Cobaleda et al., 2007a; Cobaleda et al., 2007b). Pax5 is exclusively expressed in B-lineage cells where it is switched on during pre-pro-B to pro-B cell transition and its expression remains steady until mature B cell stage (Fuxa & Busslinger, 2007). Pax5 regulates the commitment by repressing B lineage inappropriate genes and by activating B cell specific genes (reviewed by Cobaleda et al., 2007b). Among the Pax5-repressed genes are myeloid-specific *c-fms* (Tagoh et al., 2006) and the lymphoid progenitor marker *Flt3* (Holmes et al., 2006). The genes whose expression are activated by Pax5 include several genes that are involved in pre-BCR and BCR signaling such as *mb-1* and *blnk* (Fitzsimmons et al., 1996; Schebesta et al., 2002; Schebesta et al., 2007) as well as the gene for the coreceptor CD19 (Kozmik et al., 1992; Nera et al., 2006; Schebesta et al., 2007). Furthermore, Pax5 promotes the expression of *EBF1* (Nera et al., 2006; Roessler et al., 2007). In addition to B cell commitment, Pax5 is also needed for maintaining the B cell program. Deletion of Pax5 at later stages of B cell development leads to a loss of mature B cells and either dedifferentiation into progenitors that regain developmental plasticity or induce further differentiation into plasma cells (Horcher et al., 2001; Mikkola et al., 2002; Nera et al., 2006; Cobaleda et al., 2007a).

EBF1 seems to have similar role to Pax5 in the commitment to B lineage and B lineage maintenance. $EBF1^{-/-}$ progenitor cells, even when pre-cultured in conditions promoting B lymphopoiesis, have T-lymphoid and myeloid potential *in vivo* (Pongubala et al., 2008). Accordingly, ectopic EBF1 expression in MPP population produces B cells at the expense of myeloid cell fates by attenuating the expression of PU.1 and C/EBPα (Pongubala et al., 2008) that synergistically activate myeloid differentiation (Xie et al., 2004; Laslo et al., 2006; Yeamans et al., 2007). Importantly, EBF1 expression is able to rescue B cell development in $Pax5^{-/-}$ progenitors, and block the promiscuous lineage potential of $Pax5^{-/-}$ progenitor cells (Pongubala et al., 2008). Furthermore, EBF1, but not Pax5, restores the B cell development of progenitor cells deficient in PU.1, E2A or the IL-7Rα and of lymphoid progenitors isolated from IL-7-deficient mice (Medina et al., 2004; Seet et al., 2004; Dias et al., 2005; Kikuchi et al., 2005). Also, Pax5-mediated activation of B-lineage genes *CD19* as well as *mb-1* depend on EBF1 expression (Maier et al., 2004; Medina et al., 2004). Thus, as Pax5 promotes EBF1 expression (Nera et al., 2006; Roessler et al., 2007), the developmental plasticity of $Pax5^{-/-}$ cells may be partly due to reduced EBF1 expression. Despite the

fact that EBF1 regulates Pax5 expression (O'Riordan & Grosschedl, 1999; Medina et al., 2004), EBF1 can promote the B cell fate commitment independently of Pax5 by repressing alternative lineage options and promoting B cell specific gene expression program (Pongubala et al., 2008).

Enforced ectopic expression of EBF1 in *Ikaros*$^{-/-}$ LSK cells restores the generation of CD19^{+} pro-B cells (Reynaud et al., 2008), that are missing in *Ikaros*$^{-/-}$ mice (Wang et al., 1996), underlining the importance of EBF1 for the generation of B cell progenitors. However, these cells are not committed to the B lineage, despite having normal level of EBF1 and Pax5 expression (Reynaud et al., 2008). This finding suggests that in addition to Pax5 and EBF1, also Ikaros regulates the commitment to B lineage. *Ikaros*$^{-/-}$ progenitor cells cannot undergo V_H to DJ_H recombination either, as Ikaros promotes directly the recombination activating gene (Rag) expression and regulates IgH locus compaction and accessibility of the variable gene segments (Reynaud et al., 2008).

E2A is required for the expression of EBF1 and Pax5 and the B cell specific program in pro-B cells (Kwon et al., 2008). Recent findings also suggest an important role for E2A in maintaining the B cell program, as conditional inactivation of E2A reduces pro-B cell, pre-B cell, immature B cell and germinal center B cell development (Kwon et al., 2008; Beck et al., 2009). E47 is necessary for developmental progression beyond the pro-B cell stage (Beck et al., 2009), but both E12 and E47 are required for V_LJ_L gene rearrangement in pre-B cells and Igλ gene transcription in immature B cells (Beck et al., 2009).

Thus, the commitment to B lineage is guided by a network of transcription factors where EBF and Pax5 are central players but also other transcription factors are known to contribute (Reynaud et al., 2008; Lin et al., 2010).

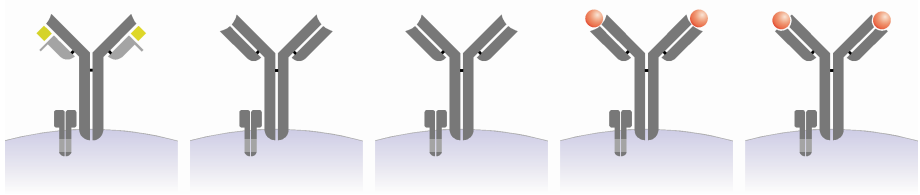
2.2 BCR signaling guides cell fate decisions and function of B cells

The purpose of the B cell lineage development is to provide a specific recognition of pathogens. Therefore, a variety of BCR specificities have to be generated without prior experience of the pathogens. To succeed, B cells have to generate a maximal variety of specificities while avoiding self recognition. To escape the limited genetic information, the B cells create the genetic information *de novo*, by rearranging several copies of immunoglobulin (Ig) gene segments with considerable sloppiness (junctional diversity). To monitor the functionality of each rearrangement event and to allow self tolerance, the newly rearranged genes are expressed as immunoglobulin on the cell surface and probed for signaling strength at different stages during the development (Table 1, reviewed by Niiro & Clark, 2002; Tussiwand et al., 2009; Kurosaki et al., 2010). At the pre-B cell stage the Ig heavy (IgH) chain is expressed with surrogate light chains (SLC) λ5 and VpreB and together with signal transducing components (Igα and Igβ) form the pre-BCR. At the immature stage (Figure 4 and Table 2), the SLC is replaced by either κ or λ light chains. The basal level of signaling through pre-BCR or BCR constitutes a survival signal for the cell (Shaffer & Schlissel, 1997; Kraus et al., 2004; Monroe, 2006). If the signal becomes too strong and/or too early in the development, i.e. when a ligand is bound, the cells undergo apoptosis or further rearrangement, a process called receptor editing (reviewed by von Boehmer & Melchers, 2010). The monitoring events at different developmental stages can be

considered as checkpoints that guide the cell fate as they can either promote the survival of the cells, initiate further differentiation or induce apoptosis (Table 1).

Immunoglobulin gene rearrangement is initiated by the Rag1-Rag2 protein complex that generates double stranded DNA breaks between the recombination signal sequences and gene segments (Schatz et al., 1989; Oettinger et al., 1990). The ends of the DNA molecules are then reconnected by a DNA repair process by proteins of the non-homologous end joining (NHEJ) system (Lieber et al., 2003; Rooney et al., 2004).

Table 1 | Signaling through pre-BCR and BCR have important functions at several stages of B cell development



	Checkpoint 1	Checkpoint 2	Checkpoint 3	Activation	Post GC differentiation
Place	Bone marrow	Bone marrow	Spleen	Primary follicles	Germinal centers
Stage	Pre-B to immature	Immature to transitional	Immature T1 and T2 to mature	Naïve to affinity matured	Affinity matured
Receptor	Pre-BCR	BCR	BCR	BCR	High affinity BCR
Check	Functional IgH ?	Functional IgL and BCR ? Autoreactivity ?	Autoreactivity ?	Antigen binding ?	High affinity ?
Signal	Proliferation	Weak tonic: survive Strong (auto-antigen): apoptosis or receptor editing	Weak tonic BCR + BAFF-R: survive Strong: apoptosis or anergy	Affinity maturation: proliferation and SHM	Strong BCR + CD40: stop proliferation and start differentiation
Antigen	Independent	Independent	Independent	Dependent	Dependent
Ligand	Negatively charged molecules	No ligand (tonic) or autoantigen	No ligand (tonic) or autoantigen	Antigen	Antigen

2.2.1 Pre-B stage

Pre-pro-B (fraction A) cells start to express the Rag genes (Figure 4). The early pro-B cells (fraction B), start to rearrange their immunoglobulin heavy chain gene D segment to J segment (D to J_H) and at the late pro-B cell stage (fraction C) the cells begin to undergo V_H to DJ_H rearrangement. IL-7R signaling continues to be important in committed B cells before the acquisition of a functional pre-BCR. Pro B cells are the first B lineage cells that express Igα and Igβ on the cell surface and at this stage they start to express CD19, marking the earliest progenitors committed to B lineage (Figure 4, Hardy et al., 2007).

Direct progeny of these cells are the large pre-B (fraction C′) cells that have lost their c-Kit expression and have undergone V_H to DJ_H rearrangement in their μ heavy chain locus (Figure 4). This developmental stage constitutes an important

developmental checkpoint (Table 1). The primary function of this checkpoint is to monitor, whether IgH gene has rearranged successfully and whether it is capable of forming a signaling competent pre-BCR by expressing the Igμ from the newly rearranged heavy chain gene together with the invariant surrogate light chain (SLC) proteins λ5 and VpreB (reviewed by Herzog et al., 2009). The signaling triggers heavy chain allelic exclusion by downregulating *Rag1* and *Rag2* (Grawunder et al., 1995), proliferation of V_HDJ_H-rearranged pre-B cells (clonal expansion), and terminates SLC expression (Parker et al., 2005; Thompson et al., 2007), thereby limiting proliferation of the cells, and allowing subsequent development. In the large cycling pre-B cells Ikaros and EBF1 compete in the regulation of λ5 expression, which as a component of pre-BCR drives the cell proliferation (Sabbattini et al., 2001; Thompson et al., 2007). Aiolos expression increases rapidly when the pre-BCR is downregulated and together with Ikaros overcome the effect of EBF1 and finally halts the expression of λ5 and downregulate c-Myc to prevent proliferation (Thompson et al., 2007; Ma et al., 2010)

The pre-BCR signals the cells to undergo IgH chain gene allelic exclusion, proliferative expansion and Igκ locus activation and V_κ to J_κ recombination. Mice that lack surrogate light chains fail to undergo proliferative expansion, but can survive and differentiate (Rolink et al., 2000; Hess et al., 2001). The nature of the initiating signal for pre-BCR is controversial, but several studies have shown that the non-immunoglobulin tail of λ5 has a critical role (Vettermann & Jack, 2010). The basal or tonic signaling may be initiated by self aggregation of receptor molecules through the λ5 and VpreB tail interactions. Arginine-rich tail of λ5 may also promote aggregation by helping the pre-BCR to interact with negatively charged ligands such as self antigens on stromal cell surface, DNA, etc. (Bradl et al., 2003; Ohnishi & Melchers, 2003; Bankovich et al., 2007).

The mechanism of signaling from pre-BCR is less well characterized than the signaling through BCR. However, available data suggests, that both receptors use the same main signaling pathways (Figure 5, Guo et al., 2000). For the feedback signal of the productive IgH gene recombination, the intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) of the Igα or Igβ (Fuentes-Panana et al., 2006; Storch et al., 2007) as well as Src and Syk family protein tyrosine kinases are required (Saijo et al., 2003). As demonstrated by Lyn/Fyn/Blk triple-deficient pro-B cells that have abolished pre-BCR mediated NF-κB activation, Src family tyrosine kinases Lyn, Fyn and Blk contribute to the proliferative expansion in a redundant fashion (Saijo et al., 2003). Lyn and Syk phosphorylate the ITAMs of the Igα and Igβ (Sanchez et al., 1993; Flaswinkel & Reth, 1994). Syk-deficiency leads to a developmental block in the pre-B cell stage and Syk-deficient B cells fail to undergo clonal expansion (Cheng et al., 1995; Turner et al., 1995). Downstream of Syk activation, Ras activates ERK1 and ERK2, which is needed for pre-BCR mediated cell expansion through Elk1 and CREB transcription factors (Yasuda et al., 2008). It has also been hypothesized that Syk-mediated activation of PI3K-PKB pathway leads to inhibition of Foxo transcription factors that eventually promote proliferation, since Foxo regulates the CDK inhibitor p27 (Dijkers et al., 2000; Medema et al., 2000; Nakamura et al., 2000; Kops et al., 2002).

Syk is also needed for the pre-BCR signaling to facilitate the IgL recombination. Important substrate for Syk in this process is BLNK (SLP-65, BASH). Syk

phosphorylates BLNK at multiple tyrosine residues, that once phosphorylated, serve as docking sites for Src-homology 2 (SH2) domain containing signaling proteins, such as BTK, PLCγ2 and Grb2, leading to an assembly of a macromolecular signaling complex (Su et al., 1999; Guo et al., 2000; Chiu et al., 2002). BLNK mediates the pre-BCR signaling that terminates the expression of SLC and mediates the upregulation of Igκ gene recombination (Parker et al., 2005; Thompson et al., 2007). With a feedback of tonic pre-BCR signal, the checkpoint is passed (Table 1).

As there is synergism between IL-7R and pre-BCR in activation of cell proliferation and pre-B cells still express IL-7R, the proliferation of early B cells is influenced also by IL-7 (Marshall et al., 1998; Fleming & Paige, 2002; Storch et al., 2007). IL-7R signals through Jak1/Jak3 to activate Stat5 (reviewed by Paukku & Silvennoinen, 2004). Before the pre-B cell stage, the IL-7R-Stat5 signaling keeps the transcription factors B cell lymphoma 6 (Bcl6) repressed (Duy et al., 2010). After productive V_H to DJ_H gene rearrangement the pre-BCR signaling downregulates the IL-7 responsiveness and leads to Stat5 dephosphorylation by several possible mechanisms (Schebesta et al., 2002; Johnson et al., 2008; Nakayama et al., 2009). Loss of Stat5 signaling in pre-B cells induces high expression of Bcl6 that protects pre-B cells from DNA-damage-induced apoptosis as a result of IgL chain gene rearrangement (Duy et al., 2010) as demonstrated in germinal centers (see later).

The mechanism of initiation of light chain gene rearrangement is not entirely understood, but several models have been proposed (Herzog et al., 2009). Foxo1 appears to be involved in IgL chain recombination by promoting the transcription of *Rag*s (Amin & Schlissel, 2008; Herzog et al., 2008).

The expression of pre-BCR on the cycling large pre-B cells induces the PI3K-Akt pathway that inhibits Foxo1 function. Foxo proteins can activate Rag gene expression (Amin & Schlissel, 2008; Herzog et al., 2008) and entry into small-pre-B cell stage. BLNK activates the PLCγ2 pathway downstream of pre-BCR (Taguchi et al., 2004). In the absence of BLNK most cells remain proliferative. However, when BLNK is present, the signaling through Syk-PI3K-PKB-pathway is downregulated, the inhibition of Foxo1 is released allowing Foxo1 to participate in IgL gene recombination (Dengler et al., 2008) and the cells become small pre-B cells, and differentiate further into immature B cell stage, which functions as a second checkpoint (Table 1).

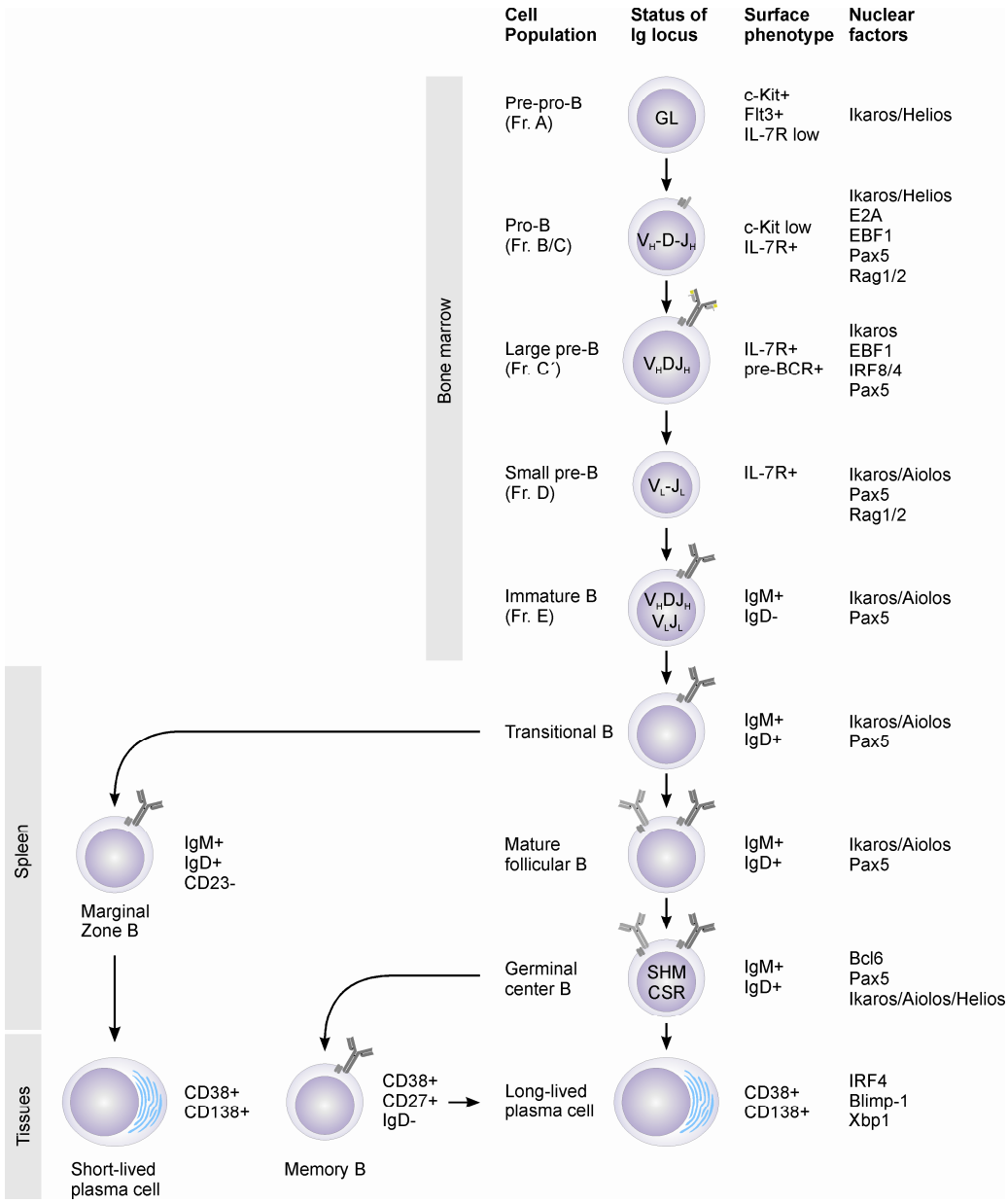


Figure 4 | Development of B cells and differentiation into plasma cells
Development of B cells into high-affinity antibody-producing plasma cells is orchestrated according to immunoglobulin gene rearrangement in the bone marrow. Undergoing gene rearrangement is indicated with a dash (-). The BCR-dependent development takes place in the periphery. The differentiation into high-affinity antibody-producing plasma cells is initiated in the GCs after SHM and CSR. The plasmablasts find their niche in the bone marrow, where they differentiate into long-lived plasma cells. Post-germinal center B cells can also differentiate into memory B cells that can be rapidly induced to produce plasma cells. Development via marginal zone gives rise to short-lived plasma cells. The characteristic surface phenotype of each stage is indicated next to the cell representing the developmental stage and the nuclear factors that are expressed or have key roles during the development into the high-affinity plasma cells are inidicated on far right. GL, germline Ig gene configuration.

2.2.2 Immature B stage

Immature B cells are the first B lineage cells that express BCR on the cell surface. This developmental stage constitutes the second significant checkpoint in B cell development (Table 1). Similarly to the pre-BCR, the BCR on the immature cells is capable of ligand independent basal (tonic) signaling that is required for survival positive selection of B cell clones (Wienands et al., 1996; Lam et al., 1997; reviewed by Monroe, 2006). This checkpoint monitors the success of IgL gene rearrangement and is important for tolerance to self structures.

Several experiments have shown that the strength of the BCR signal determines whether cells are allowed to progress in development or will undergo receptor editing to be rescued from apoptosis (reviewed by Tussiwand et al., 2009). If the IgH and the newly rearranged IgL pair well and express high levels of BCR, the tonic signaling is achieved. These cells turn off their *Rag* expression and are allowed to migrate to spleen for further development (positive selection). Those cells that express a non-autoreactive but poorly interacting IgH-IgL pair cannot maintain adequate signaling. Lack of tonic BCR signaling traps the developing B cell to a stage where they undergo secondary recombination events. Reducing the level of tonic signaling by conditional receptor knockout or use of inhibitors to block BCR-induced pathways results in an increased Rag expression and new IgL recombination as well as expression of other pro- and pre-B cell associated genes (Keren et al., 2004; Tze et al., 2005,). If the pre-B cells succeed in generating a receptor that gives appropriate tonic signals, the cells can develop further. The cells that express an autoreactive BCR, the receptor is ligated. This inappropriately strong signaling induces either apoptosis to delete the autoreactive clone, or induces anergy (reviewed by Gauld et al., 2006), where B cell remains non-responsive. Also autoreactive B cell clones can be rescued by receptor editing, where the specificity of the receptor is altered by continued rearrangement of immunoglobulin gene segments (Chen et al., 1995). Regulation by Foxo1 has been suggested to participate in this process (Amin & Schlissel, 2008).

2.2.3 Peripheral B cells

In the development of the conventional B2 B cells, the immature cells emigrate from the bone marrow to spleen and are then referred to as transitional 1, 2 and 3 (T1, T2 and T3) B cells and can be distinguished based on their surface marker expression. T1 stage precedes the T2 stage and the T2 cells are considered the direct progeny to mature naïve B cells. Transitional type immature B cells are still sensitive to IgM-induced apoptosis (Carsetti et al., 1995; Rolink et al., 1998; Loder et al., 1999; Rolink et al., 1999a; Allman et al., 2001) and *in vivo* studies have indicated that negative selection against autoreactivity may take place also at this stage (Wardemann et al., 2003; Meffre & Wardemann, 2008). Identity of T3 cells is less clear but they are suggested to be anergic B cells (Merrell et al., 2006).

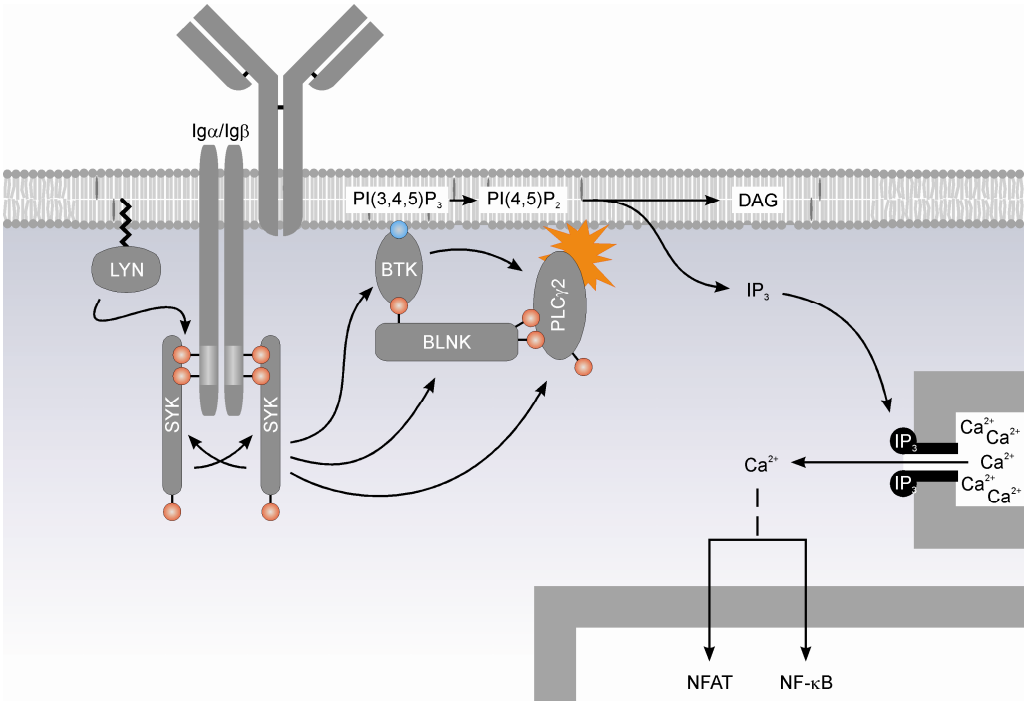


Figure 5 | Assembly of BCR signalosome and induction of PLCγ2 pathway
Association of pre-BCR or BCR with active Src-family kinase Lyn upon activation, results into phosphorylation of Igα and Igβ in immunoreceptor tyrosine-based activation motif (ITAM) tyrosines, that create docking sites for spleen tyrosine kinase Syk SH2-domain. Syk is activated by binding to phospho-ITAM and phosphorylation by Lyn, resulting in an increased phosphorylation of ITAMs and further Syk activation. Syk phosphorylates and activates Bruton's tyrosine kinase Btk that is brought to lipid raft by the interaction between its PH domain and $PI(3,4,5)P_3$ lipid within the plasma membrane. Syk also phosphorylates BLNK that helps to recruit phospholipase C-γ2 (PLCγ2) to the membrane. Phosphorylation of PLCγ2 by Btk and Syk activates PLCγ2, which then cleaves $PI(4,5)P_2$ to yield diacylglycerol (DAG) and inositol trisphosphate (IP_3) that opens a calcium channel (IP_3 receptor) on endoplasmic reticulum to allow increase in intracellular calcium concentration. Calcium activates several molecules, such as calmodulin and, together with DAG, protein kinase C, eventually leading to activation of downstream transcription factors such as NF-κB and NFAT.

Tonic BCR signaling is important also for development of T1/T2 B cells to mature B2 B cells. Ablation of BCR signaling by conditional inactivation of IgM prevents development beyond transitional stage (Kraus et al., 2004). Similarly, deletion of genes *Btk* (Khan et al., 1995), *Blnk* (Pappu et al., 1999), *Pik3ap1* (Yamazaki et al., 2002), *Plcg2* (Hashimoto et al., 2000) or *Vav1*/*Vav2*/*Vav3* (Fujikawa et al., 2003; Vigorito et al., 2005) that are involved in signal propagation from BCR (Figure 5), have also insufficient signaling to induce mature B cell differentiation from transitional stage. These molecules activate the NF-κB pathway, whose importance for B cell maturation is also highlighted by deletion of CARMA1 or IKKγ (reviewed by Schulze-Luehrmann & Ghosh, 2006)

In addition to BCR signals, transitional stage B cells require B cell activating factor (BAFF)-mediated signals for survival and full maturation. BCR ligation upregulates the expression of BAFF-receptor, increasing the sensitivity of B cells to BAFF (Smith & Cancro, 2003; Stadanlick et al., 2008; Castro et al., 2009). The effects of BAFF are critical for T2 B cells as BAFF and BAFF-receptor deficient mice have a block at the T1 stage of the B cell development (Schiemann et al., 2001; Yan et al., 2001; Sasaki et al., 2004; Shulga-Morskaya et al., 2004; Swee et al., 2010). BAFF-signaling is also important for tolerance, as mice overexpressing BAFF develop lupus-like disease (Mackay et al., 1999; Batten et al., 2000; Gross et al., 2000; Khare et al., 2000). In addition to participating in BCR signaling pathway, PLCγ2 is also active in BAFF-receptor signaling (Hikida et al., 2003).

2.2.4 Activation of B cells

After binding protein antigens, the mature naïve B2 B cells move to T cell zones of the secondary lymphoid tissues along a chemokine CCL21 gradient to receive T cell help. The B cell either differentiates along the follicular pathway to generate germinal centers or proceed to an extrafollicular pathway, which creates short-lived plasma cells producing low affinity antibodies. The extrafollicular pathway is also initiated in a response against type II antigens that usually contain repeating antigen determinants on a large polysaccharide backbone, and sustains plasmablasts in regions where $CD11c^{high}$ DC cells provide APRIL and BAFF such as splenic extrafollicular foci and lymph node medullary cords (reviewed by MacLennan & Vinuesa, 2002).

The expression of transcription factor Aiolos is restricted to the lymphoid cells and is highest in mature peripheral B cells (Morgan et al., 1997; Koskela et al., 2003). The expression of Aiolos is directly controlled by Ikaros, NF-κB and AP4 (Ghadiri et al., 2007). Peripheral B cells of Aiolos-deficient mice have activated cell surface phenotype and generate germinal centers spontaneously, suggesting a role for Aiolos in setting a threshold for BCR activation (Wang et al., 1998; Cariappa et al., 2001). Aiolos-deficient mice have normal low affinity plasma cell development before the spontaneous germinal center development and are defective in selecting of somatically hypermutated germinal center B cells (Cortes & Georgopoulos, 2004). Aiolos activates Bcl-2 in T cells and interacts with Bcl-X_L and can prevent apoptosis (Romero et al., 1999; Rebollo et al., 2001). Accordingly, Aiolos-deficient B cells have an apoptosis-prone phenotype (Narvi et al., 2007).

2.3 Germinal centers

Germinal center (GC) formation is central for effective acquired immunity. Germinal centers are transient structures that typically arise in T-dependent B cell responses during the first 3 weeks after antigen exposure providing appropriate environment for affinity maturation of immunoglobulin molecules and for the change of immunoglobulin effector class (MacLennan, 1994). These features are manifestations of somatic hypermutation (SHM) coupled to positive selection and class-switch recombination (CSR). As the germinal center response includes rapid B cell proliferation and active genomic mutations, GC B cells are likely candidates for malignant transformation. Indeed the majority of B cell lymphomas originate from GC

B cells (reviewed by Stevenson et al., 1998; Kuppers et al., 1999; Klein & Dalla-Favera, 2008).

To initiate germinal center response, the B cells need coactivating signals from T cells and/or dendritic cells through B cell surface receptors, such as CD40. CD40 ligand (CD40L, CD154) is expressed by follicular T helper cells. In the response to these signals activated B cells move to primary follicle, the B cells start intensive proliferation and form a secondary follicle. In fully matured GC, a dark zone consisting of densely packed proliferating B cells, called centroblasts, and a light zone where non-dividing B cells, called centrocytes are evident (Figure 6). The centrocytes reside in a mesh formed by follicular dendritic cells (FDCs). The centroblasts start to mutate their immunoglobulin heavy and light chain gene hypervariable regions in SHM. Those B cell clones that have increased affinity to antigen are selected in the light zone. The dark-zone-light-zone-pattern is not always well defined and antigen-specific B cells migrate within and between light and dark zones *in vivo* (Camacho et al., 1998; Wang & Carter, 2005; Allen et al., 2007; Hauser et al., 2007; Schwickert et al., 2007). The movement is possibly guided by stromal cell derived chemokines CXCL12 and CXCL13 originating from light zones and dark zones, respectively (Allen et al., 2004).

The centroblasts are one of the most rapidly proliferating cell types in the body. Gene expression profiling experiments have revealed that they upregulate genes associated with cell proliferation and downregulate the expression of genes that inhibit cell division (Shaffer et al., 2001; Klein et al., 2003). The centroblasts are also inherently prone to apoptosis. GC B cells die rapidly *in vitro* unless they are rescued by anti-apoptotic signals (Liu et al., 1989; Feuillard et al., 1995; Billian et al., 1996) and centroblasts lack anti-apoptotic factors such as Bcl-2, while they upregulate pro-apoptotic molecules including FAS and p53 (Liu et al., 1991; Martinez-Valdez et al., 1996; Klein et al., 2003).

In accordance with the apoptosis prone phenotype of GC B cells, the centroblasts do not activate the anti-apoptotic NF-κB pathway, as they do not express NF-κB target genes or exhibit NF-κB activation (Shaffer et al., 2001). CD40L-CD40 signaling would activate NF-κB pathway, but centroblasts do not seem to have active CD40 signaling either, as the genes activated by CD40 signaling are not expressed (Basso et al., 2004). This is consistent with the view that the dark zone is devoid of CD40L carrying cells such as T cells (MacLennan, 1994; Klein & Dalla-Favera, 2008). However, a subset of centrocytes have nuclear location of NF-κB (Basso et al., 2004) and disruption of CD40-CD40L-interaction results in dissolved GCs (Han et al., 1995) suggesting a role for CD40 in the light zone, where it may provide survival signals for B cells exiting germinal centers (Klein & Dalla-Favera, 2008). This theory is supported by the finding that CD40-mediated NF-κB activation in GCs upregulates the IRF4 expression, which in turn represses Bcl6 (Saito et al., 2007), the transcription factor required for germinal center formation (Dent et al., 1997; Ye et al., 1997) and allows further differentiation.

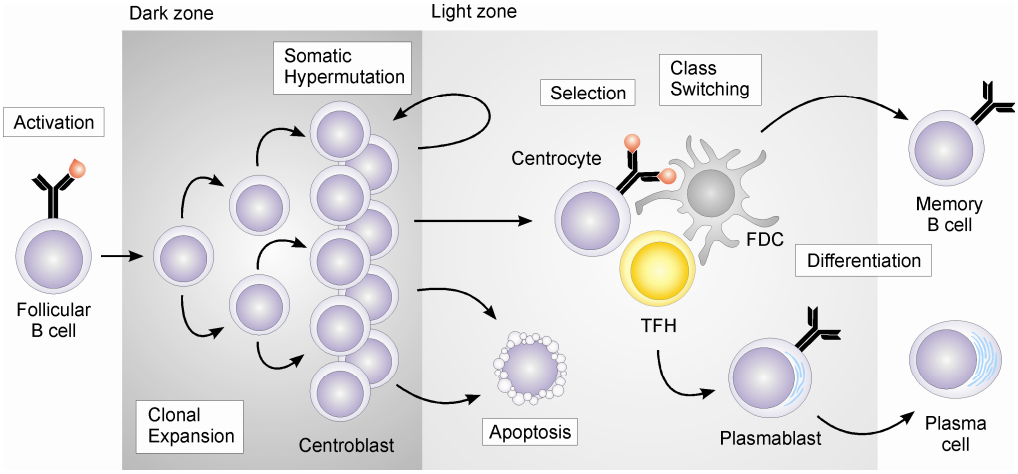


Figure 6 | Course of events in germinal center
Activated B cells differentiate into centroblasts and start proliferation and introduction of point mutations by somatic hypermutation (SHM) into variable region of the rearranged immunoglobulin heavy and light chain genes. Centroblasts migrate to light zone of the germinal center where they are tested for increased affinity of the BCR. Most cells have not acquired increased affinity for antigen and some may have acquired autoreactivity. These cells either undergo more cycles of proliferation and SHM or die by apoptosis, since mutations that prevent antigen binding, result in signaling that does not support survival. Cells in the GC such as follicular helper T cells (T_{FH}) and follicular dendritic cells (FDC) select those few clones that have acquired increased affinity for antigen. Some centrocytes undergo class switching to change the immunoglobulin effector class. Positively selected high-affinity centrocytes are selected for further differentiation into plasmablasts and eventually into plasma cells in the tissues or differentiate into memory B cells that are capable of eliciting a fast response if the cognate antigen is encountered again.

2.3.1 Somatic hypermutation and class-switch recombination

Another striking feature of centroblasts is that they allow DNA damages (Phan & Dalla-Favera, 2004). This property may be useful to accomplish SHM, a basis for affinity maturation that primarily takes place in centrocytes of the germinal centers (Berek et al., 1991; Jacob et al., 1991; Kuppers et al., 1993).

Somatic hypermutation modifies the immunoglobulin variable region in the centroblasts and occurs at a rate of 10^{-3} mutations per base pair per cell generation. Activation-induced cytidine deaminase (AID) marks immunoglobulin variable gene regions for somatic hypermutation by deaminating cytidine in the C-4 position of the pyrimidine base yielding a uridine. The U-G mismatch in the DNA may give rise to mutations by different mechanisms (Figure 7). Replication of the DNA gives rise to C to T or G to A mutations (transitions). Recognition of the U-G mismatch by Msh2/Msh6 mismatch repair heterodimer leads to excision of a stretch of DNA and the error-prone repair of the gap, resulting in the spreading of mutation (Figure 7). However, the most commonly used pathway is through base excision repair (BER). The uridine in the DNA is a substrate for uracil-DNA glycosylase (UNG) that removes uracil and leaves an abasic site to DNA (Figure 7, Di Noia & Neuberger, 2002; Rada et al., 2002). This lesion is converted into a single-stranded break by apurinic/apyrimidic endonucleases (APE1 and APE2) and is repaired by error-prone polymerases, leading

eventually into both transversions and transitions (for a detailed discussion of the mechanism, see Di Noia & Neuberger, 2007; Peled et al., 2008)

Interestingly, SHM has been reported also to occur outside of Ig variable loci, including *Bcl6* locus (Pasqualucci et al., 1998; Shen et al., 1998; Muschen et al., 2000; Gordon et al., 2003; Liu et al., 2008). No direct targeting mechanisms of AID to act specifically on certain genes have been so far identified, suggesting that genes for AID-induced mutations are selected at a different level. Indeed, it seems that AID acts widely on the genome to deaminate cytidines on several genes and induces DNA lesions (Liu et al., 2008; Mahowald et al., 2008; Hasham et al., 2010). The final outcome of genes that are mutated seems to rely on the balance between high-fidelity and error-prone repair mechanisms (Figure 7, Liu et al., 2008; Hasham et al., 2010).

The initiation of CSR utilizes largely the same mechanisms as SHM (Figure 7), particularly it is initiated by AID to generate the U-G mismatch and proceeds along the BER pathway including UNG and APE. However, it results in the double-stranded breaks in the switch regions that are joined to switch regions of other functional class of Ig heavy chain genes by ligase IV (reviewed by Stavnezer et al., 2008).

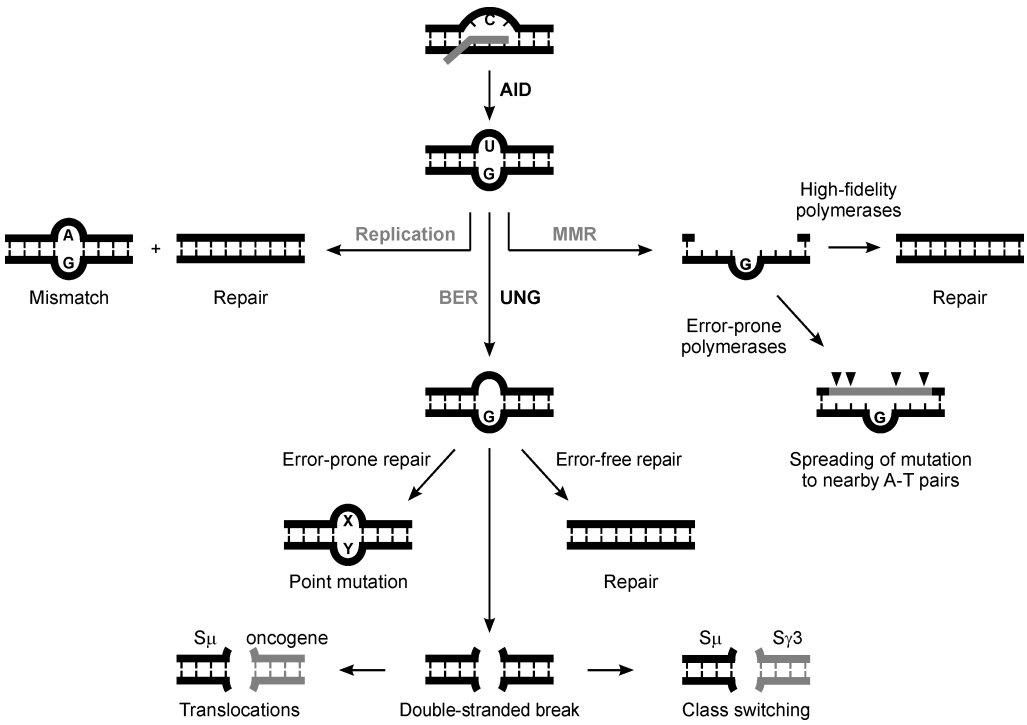


Figure 7 | A model for AID-induced lesion repair
Somatic hypermutation (SHM) and class-switch recombination (CSR) are initiated by AID-mediated cytidine deamination. The generated U-G mismatch is subject to replication, mismatch repair (MMR) pathway or base-excision repair (BER) pathway. In BER pathway UNG creates an abasic site that is repaired by an error-free mechanism resolving the lesion or by error-prone mechanism that can induce a point mutation. Abasic site can also be cut by AP-endonuclease creating a single-stranded break that can either be repaired correctly or in an error-prone fashion or result in a double stranded break. Double-stranded breaks between two switch regions result in class switching but may also cause translocations.

2.3.2 *Bcl6 regulates germinal center phenotype*

The transcription factor Bcl6 was originally identified and cloned as a gene in chromosomal translocations occurring in B cell lymphomas (Ye et al., 1993a; Ye et al., 1993b). The regulation of Bcl6 expression is complex. Within the B lineage, Bcl6 mRNA is observed in resting mature B cells as well as in activated germinal center B cells but not in plasma cells (Cattoretti et al., 1995; Allman et al., 1996). However, the expression of Bcl6 protein is highly increased in GC cells (Allman et al., 1996), with highest expression in centroblasts and lower expression in centrocytes (Kuo et al., 2007). IL-21 is shown to maintain the expression of Bcl6 and germinal centers (Arguni et al., 2006; Linterman et al., 2010; Zotos et al., 2010). IL-21 signals via Stat3 and Stat5, that are suggested to upregulate Bcl6 (Ozaki et al., 2004; Scheeren et al., 2005; Arguni et al., 2006).

Bcl6 has two domains, an N-terminal BTB/POZ domain that mediates dimerization and interaction with co-repressors and a C-terminal zinc-finger domain that mediates DNA binding (Figure 8, Baron et al., 1995; Dhordain et al., 1995; Seyfert et al., 1996). The corepressors SMRT (silencing mediator for retinoid and thyroid receptor), NCoR (nuclear receptor corepressor) and BCoR (Bcl6 corepressor) bind competitively to BTB/POZ domain (Huynh & Bardwell, 1998; Wong & Privalsky, 1998; Huynh et al., 2000; Ahmad et al., 2003; Ghetu et al., 2008). Another repressor, CtBP (C-terminal binding protein) binds to a regions that is close to and possibly overlaps with the BTB/POZ domain. Mi-2/NuRD (nucleosome remodeling and deacetylase) complex binds an unstructured region between the two domains (Figure 8, Fujita et al., 2004).

Association of Bcl6 with its co-repressor complexes is suggested to vary between distinct biological contexts. SMRT-based Bcl6 peptide inhibitor that binds to the same motif in the BTB/POZ domain than the co-repressors SMRT, NCoR and BCoR, prevents their interaction with Bcl6 (Ghetu et al., 2008). This induces expression of genes such as *ATR*, *TP53* (encoding the p53) and *CDKN1A* (the gene encoding for p21) that are involved in DNA damage and cell cycle checkpoints (Polo et al., 2004; Parekh et al., 2007; Polo et al., 2007; Ranuncolo et al., 2007; Cerchietti et al., 2008). However, association of Bcl6 with MTA3-containing Mi-2/NuRD (nucleosomes remodeling and deacetylase) complex leads to repression of genes involved in differentiation such as *Prdm1*, the gene encoding Blimp-1 (Fujita et al., 2004; Parekh et al., 2007), but the association with SMRT, NCoR and BCoR does not (Polo et al., 2004). Furthermore, interaction of CtBP with Bcl6 leads to negative autoregulation of Bcl6 expression (Mendez et al., 2008). Also zinc finger domain is involved in mediating interactions, for example to a transcription factor Miz-1 (Phan et al., 2005). Miz-1 can recruit Bcl6 to target genes that do not contain Bcl6 consensus binding site, such as *CDKN1A* (Phan et al., 2005). Several other interactions have also been described (Okabe et al., 1998; Dhordain et al., 2000; Jardin et al., 2007).

Bcl6 is as a master regulator of centroblasts. Bcl6-deficient mice, and Rag-deficient mice reconstituted with *Bcl6*$^{-/-}$ bone marrow cells, lack GCs and affinity maturation (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997). Analyses of Bcl6 target genes have suggested that Bcl6 maintains the germinal center gene expression signature that includes genes involved in maintaining extremely rapid cell proliferation and inhibiting further differentiation (Shaffer et al., 2000).

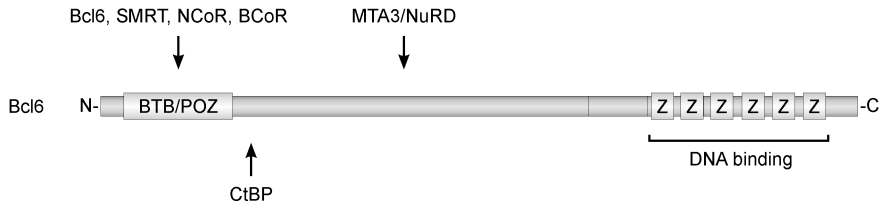


Figure 8 | Schematic presentation of domain organization of Bcl6
N-terminal BTB/POZ (bric à brac, tramtrack, broad-complex/pox virus and zinc finger) domain mediates Bcl6 dimerization and interaction with several corepressor complexes and the C-terminal zinc finger domain consisting of six zinc fingers (Z) mediates sequence-specific DNA binding. Interaction with NuRD complex has been mapped to central region (also called repression domain 2) and with CtBP to region close to and partially overlapping with BTB/POZ domain.

Bcl6 is suggested to have a couple of major functions to determine the specialized phenotype of germinal centers (Klein & Dalla-Favera, 2008). As Bcl6 represses the expression of *TP53* directly (Phan & Dalla-Favera, 2004) and the cell cycle arrest protein p21 gene *CDKN1A* through Miz-1-mediated recruitment (Phan et al., 2005), Bcl6 would allow high proliferation rate of centroblasts while the cells modify their genomes. Furthermore, Bcl6 directly represses the expression of ATR (ATM and Rad3 related), a central sensor of DNA damage (Ranuncolo et al., 2007) and suppresses the expression of CHEK1, that is activated through phosphorylation by ATR. Once activated, CHEK1 can phosphorylate p53 (Ranuncolo et al., 2008). Also SUB1, an activator of *TP53*, whose expression is increased upon DNA damage (Banerjee et al., 2004; Kishore et al., 2007) is repressed by Bcl6 (Polo et al., 2007). Thus, in addition to controlling the cell cycle arrest in response to DNA damage, Bcl6 inhibits the detection of DNA damage itself as well as the transduction of the signal to checkpoint mediators. Bcl6 protein level is regulated by phosphorylation-induced ubiquitin-mediated proteasomal degradation (Niu et al., 1998). Interestingly, ATM promotes the phosphorylation of Bcl6 in response to DNA damage, which leads to Bcl6 degradation (Phan et al., 2007), suggesting a feedback mechanism where the extent of genotoxic stress dictates the cell fate through Bcl6 levels to balance the DNA damage tolerance at levels that are physiologically appropriate in germinal centers.

Bcl6 represses also genes that are involved in T-dependent activation of B cells, such as *CD69*, *STAT1* and *CD80* (Shaffer et al., 2000; Niu et al., 2003). This suggests a role in preventing centroblast-T cell interaction before finishing proliferation and the process of somatic hypermutation.

Another important function of Bcl6 is to suppress post-germinal center differentiation that produces memory B cells and plasma cells. While B cell development is apparently normal in the absence of Bcl6 (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997) and B cells with some memory B cell functions are generated (Toyama et al., 2002), the memory B cells cannot undergo affinity maturation in the absence of Bcl6 (Toyama et al., 2002). In fact, the repression of Bcl6 seems to be required for memory B cell differentiation from germinal centers (Kuo et al., 2007).

Bcl6 is also an important repressor of post-germinal center plasma cell differentiation that can give rise to the long-lived plasma cell population (reviewed by Radbruch et al., 2006) in the bone marrow. The role of Bcl6 in the repression of plasma cell differentiation is largely attributed to its inhibiting function on the

expression of Blimp-1 (Reljic et al., 2000; Shaffer et al., 2000; Tunyaplin et al., 2004; Parekh et al., 2007). Blimp-1 is a plasma cell transcription factor that is needed to induce plasma cell differentiation and maintain the plasma cell phenotype (Turner et al., 1994; Shapiro-Shelef et al., 2003). Ectopic Bcl6 expression in primary B cells and B lymphoma cell lines inhibits plasmacytic differentiation (Reljic et al., 2000) and the expression of Bcl6 in a plasma cell line results into reactivation of a B cell gene expression program and repression of plasma cell specific transcription (Fujita et al., 2004).

2.4 Post-germinal center plasma cell differentiation

Transcriptional regulators B lymphocyte-induced maturation protein 1 (Blimp-1), interferon regulatory factor 4 (IRF4) and X-box binding protein 1 (Xbp1) are required for plasma cell differentiation (Mittrucker et al., 1997; Reimold et al., 2001; Shapiro-Shelef et al., 2003). The transcriptional program of plasma cells is driven by Blimp-1 that is necessary and sufficient for plasma cell differentiation (Turner et al., 1994; Shaffer et al., 2002; Shapiro-Shelef et al., 2003) as well as for maintenance of the long-lived plasma cells (Shapiro-Shelef et al., 2005). B cell specific deletion of *Prdm1* DNA-binding domain results in severely reduced immunoglobulin secretion (Shapiro-Shelef et al., 2003). The finding of enlarged germinal centers in these mice suggests that defective secretion is due to a developmental block at the late germinal center or post germinal center stage (Shapiro-Shelef et al., 2003). 5–15 % of GC cells are positive for *Prdm1* transcript (Angelin-Duclos et al., 2000), but high expression of *Prdm1* mRNA is not observed until Bcl6, Pax5 and Bach2 are downregulated (Kuo et al., 2007). The Blimp-1 positive GC cells do not express Bcl6 suggesting that these cells are centrocytes that have started the differentiation into plasmablasts (Angelin-Duclos et al., 2000). Indeed, Bcl6 (Tunyaplin et al., 2004), Pax5 (Mora-Lopez et al., 2007) and Bach2 (Ochiai et al., 2006) can repress *Prdm1* expression directly.

IRF4 has a two-phase expression pattern during B cell development. It is expressed in immature B cells of the bone marrow (Lu et al., 2003), but is low in proliferating centroblasts and is expressed again in some centrocytes and plasma cells (Falini et al., 2000). The low IRF4 expression in centroblasts may reflect the absence of NF-κB in these cells (Shaffer et al., 2001), since IRF4 expression is induced by NF-κB (Grumont & Gerondakis, 2000; Saito et al., 2007). In addition to its role in early B cell development, IRF4 is a critical regulator of plasma cell development (Klein et al., 2006; Sciammas et al., 2006). IRF4 deficient B cells cannot form plasma cells *in vitro* (Klein et al., 2006; Sciammas et al., 2006) and IRF4-deficient mice lack plasma cells and their serum Ig levels are low (Mittrucker et al., 1997). Also conditional inactivation *in vivo* of IRF4 blocks plasmacytic differentiation (Klein et al., 2006) and ectopic IRF4 expression promotes plasmacytic differentiation (Sciammas et al., 2006). IRF4 seems to promote the expression of *Prdm1* upon lipopolysaccharide stimulation, as IRF4 deficient mice do not induce *Prdm1* expression (Sciammas et al., 2006). Conversely, IRF4 has been suggested to function in parallel to Blimp-1 during plasma cell differentiation (Klein et al., 2006). However, the finding that IRF4 binds to *Prdm1* intronic element upon stimulation (Sciammas et al., 2006), strongly supports a model that *Prdm1* is a downstream target of IRF4. IRF4 is also found to regulate the expression of AID (Sciammas et al., 2006; Luo & Tian, 2010), the enzyme mediating

CSR (Muramatsu et al., 2000; Okazaki et al., 2002). Indeed, IRF4-deficient GC B cells fail to undergo CSR (Klein et al., 2006; Sciammas et al., 2006). Along these lines, low expression of IRF4 is suggested to induce CSR and stronger expression, after the downregulation of Bcl6, to induce plasma cell differentiation (Sciammas et al., 2006).

Xbp1 is required for plasma cell differentiation (Reimold et al., 2001), but cannot initiate the process in the absence of Blimp-1 (Shapiro-Shelef et al., 2003). During the normal plasma cell differentiation, Xbp1 operates downstream of Blimp-1 and IRF4 (Shapiro-Shelef et al., 2003; Klein et al., 2006) to regulate chaperones involved in handling the load of increased protein synthesis (Lee et al., 2003). Xbp1 expands the secretory apparatus when overexpressed in B cells and is required for antibody secretion in plasma cells (Shaffer et al., 2004). In addition to transcriptional regulation, the function of Xbp1 is controlled by differential splicing (Shen et al., 2001; Yoshida et al., 2001; Calfon et al., 2002). In the response to ER stress induced by heavily increased immunoglobulin production, activating transcription factor 6 (ATF6) is activated (Haze et al., 1999), which induces Xbp1 transcription (Yoshida et al., 2001). As a response to ER stress, inositol-requiring enzyme 1 (IRE1) splices the *Xbp1* mRNA into its functional form (Shen et al., 2001; Yoshida et al., 2001; Calfon et al., 2002).

In addition to inducing the activators of plasma cell differentiation the repressors of plasma cell differentiation need to be suppressed. Several transcription factors are involved in the suppression of plasma cell fate. A necessity for plasma cell differentiation is downregulation of Pax5 that is central in the commitment and maintenance of B cell phenotype (reviewed by Nera & Lassila, 2006). Inactivation of Pax5 gene in DT40 B cells induces a plasma cell transcription program and immunoglobulin secretion (Nera et al., 2006). Similar phenotype is observed in Pax5-deficient mice (Delogu et al., 2006). The requirement of Pax5 inhibition in the first phase of plasma cell differentiation is supported by a discovery of pre-plasmablasts, a population of germinal center B cells that have lost Pax5 expression, but not yet induced *Prdm1* expression (Kallies et al., 2007). Together these findings support a scheme where downregulation of B cell characteristics precedes the acquisition of plasma cell gene expression program. Along these lines, Pax5 can repress the expression of *Prdm1* (Mora-Lopez et al., 2007) and *Xbp1* promoter (Reimold et al., 1996) directly.

3 AIMS OF THE STUDY

1. To reveal the function of Ikaros and Helios in B cells by analyzing Ikaros- and Helios-deficient DT40 B cell lines

2. To define the molecular mechanisms for the function of Ikaros and Helios in B cells

3. To gain insight into the molecular mechanisms involved in the repression of plasma cell differentiation by creating a Bcl6-deficient DT40 B cell line

4. To define direct target genes of Bcl6 in B cells

4 MATERIALS AND METHODS

4.1 Cell culture and antibodies

Wild type chicken DT40 B cells (Baba et al., 1985) and derived mutant cell lines were cultured in RPMI 1640 medium supplemented with 10 % fetal calf serum, 1 % chicken serum, 50 μM β-mercaptoethanol, 2 mM L-glutamine, penicillin and streptomycin in 40 °C humidified atmosphere with 5 % CO_2.

Anti-PLCγ2 Ab (Ishiai et al., 1999a), anti Syk Ab (Takata et al., 1994), anti-Lyn Ab (Takata et al., 1994), anti-BLNK Ab (Ishiai et al., 1999a), anti-ChB6 mAb (L22) (Pink & Rijnbeek, 1983) were described previously. Anti-Ikaros Ab (E-20), anti-Cbl Ab (C-15), anti-SHIP-1 (V-19), anti-Pax5 mAb (A-11), anti-phosphotyrosine mAb (PY99) and HRP conjugated anti-mouse IgG and anti-goat IgG Abs were from Santa-Cruz Biotechnology Inc. Anti-chicken IgM mAb (M4 and M1), anti-chicken λ light chain mAb (L1) and anti-CD45 mAb (LT40) were from Southern Biotechnology Associates Inc. Anti-Flag mAb (M2) was from Sigma.

4.2 Generation of mutant DT40 B cell lines

4.2.1 Generation of Ikaros knockout DT40 B cell line

Ikaros gene was inactivated in the DT40 cell line with gene targeting vectors Ik-bsr, Ik-neo and Ik-pur consecutively. The vectors were generated by flanking selection cassettes by homologous arms of 0,4 kb in the 5´ side and 0,7 kb in the 3´ side. The 5´ arm was amplified from DT40 genomic DNA with primers 6Lf (AAAGGTACCGAC-TAGCAAGTAACGTCGCCTAACGTAAG) and 7Lr (AAAGGATCCATCTTATGG-AAGATCAGATAGTCACTTCTCAC) that created *Acc*65I and *Bam*HI sites, respectively. The 3´ arm was amplified from DT40 cDNA using primers 7Rf (AAA-GGATCCAACTAAGAGAAGGAGAACTAGATAATGCAG) and 7Rr (AAAGTC-GACTTAACTCATGTGGAAACGGTGCTCCCCTCGAGT) that amplified a sequence from exon 7 and created *Bam*HI and *Sal*I sites, respectively. Both arms were transferred to pUC18 vector into corresponding sites and selection marker genes from LoxP vectors (Arakawa et al., 2001) were cloned into a *Bam*HI site between the arms. The resulting Ik-bsr, Ik-neo and Ik-puro vectors were linearized with *Acc*65I and introduced sequentially into DT40 cells by electroporation (710V, 25μF). Transfected clones were selected in the presence of blasticidin S (50 μg/ml), G418 (2 mg/ml) or puromycin (0,5 μg/ml) for Ik-bsr, Ik-neo and Ik-puro transfections, respectively. After each transfection, the targeting was monitored by PCR using selection marker specific primer (either Br (CGATTGAAGAACTCATTCCACTCAAATATACCC) for Ik-bsr, Nr (GCGCATCGCCTTCTATCGCCTTCTTGACGAG) for Ik-neo or Pr (CAGCGC-CCGACCGAAAGGAGCGCACGACC) for Ik-puro) together with *Ikaros* specific primer 6f (AACTAACCAGAGTGAAATGGCTGAAGACCTG). PCR products were probed in Southern hybridization using *Ikaros* specific probe 6p (CCTGTGCAAGAT-AGGGTCAGAAAGATCCCTCG).

4.2.2 Genetic complementation of Ikaros expression

The full length Ikaros isoform Ik-1 was amplified from DT40 cDNA using primers Ik-fN (AAAGCTAGCATGGAAACAGATGAGGCTCAAGA) and Ik-rN (ATTGCTAG-CTTAACTCATGTGGAAACGGTGCT). These primers created *Nhe*I sites that were used to clone the PCR product into corresponding site of pExpress vector (Arakawa et al., 2001). The resulting vector has a coding sequence for Ik-1 driven by chicken β-actin promoter with SV40 poly-a signal. This expression cassette was excised with *Spe*I and ligated into SpeI site of pLoxHisD (Arakawa et al., 2001). The expression vector with histidinol dehydrogenase selection marker was linearized with *Not*I and transfected to *Ikaros*$^{-/-}$ DT40 B cell line with electroporation and transfected clones were selected in the presence of 1,5 mg/ml histidinol. Expression of Ikaros was verified with western blotting.

4.2.3 Generation of Helios knockout DT40 B cell line

Helios gene was targeted with Hel-neo and Hel-bsr vectors that have selection cassettes flanked by chicken Helios sequence on the 5′ and 3′ sides. The 5′ arm of the vector was amplified from DT40 genomic DNA with primers H6Lf (AAAGGATCCTAT-GGAAGATTGTAAGTAAACAAGAGCCTGTGA) and H7Lr (AAAGGATCCAGT-GAATATCTGGTTAGCCAAGTCTCATGAGCT) to give 4 kb stretch of genomic sequence ranging from exon 6 to exon 7 of the Helios gene. The primer sequences contain *Acc*65I and *Bam*HI sites that were incorporated into the 5′ and 3′ ends of the PCR product, respectively. The primer sequences also introduced stop codons to both ends of the arm. *Acc*65I and *Bam*HI sites were used to transfer the homologous arm into pUC18 vector. The 3′ arm was created from DT40 cDNA using primers H7Rf (AAAGGATCCTATGAGTAAGAGTCTGAGCTGATACAGT) and H7RrUTR (AAAGTCGACTGAAACCCCACAGCATATCTGCACATATGA) designed for the Helios exon 7 sequence. These primers introduced *Bam*HI and *Sal*I sites into 5′ and 3′ ends of the arm, respectively. These sites were used to transfer 3′ arm into corresponding sites of pUC18 vector containing the 5′ arm. The selection marker expression cassettes were cloned into *Bam*HI site between the two arms in the pUC18 vector. The vectors were linearized with *Acc*65I digestion. The transfection and selection of clones were done as described in generation of *Ikaros*$^{-/-}$ cell line with the corresponding markers. The targeting was monitored after each transfection event with genomic PCR using primer 5f (CCTCACAAGTGCAACTACTGTGGCCGGAGCTA) specific for the chicken Helios exon 5 together with Nr (for Hel-neo) and Br (for Hel-bsr).

4.2.4 Genetic complementation of Helios expression

The full-length Helios coding sequence was amplified by PCR from cDNA prepared from bursa of Fabricius of chicken embryos at the 13th day of embryonic development (a gift from Dr. T. Uchida). Primers Helios-f (TATACTAGTATGGAAGCAGAG-GCTGCTGATGGATA) and Helios-r (TATGGATCCCTAGTGGAATGTGTGCTC-CCCTCGAA) that were used for amplification created *Spe*I and *Bam*HI sites, respectively. These sites were used to transfer Helios protein coding sequence between *Nhe*I and *Bgl*II sites in the pExpress vector (Arakawa et al., 2001) and subsequently the expression cassette containing β-actin promoter, Helios coding sequence and SV40

poly-A signal was transferred as SpeI cassette to pLoxPuro vector (Arakawa et al., 2001). The vector construct was linearized with *Not*I and transfected to *Helios*$^{-/-}$ cells and selected in the presence of 0,5 μg/ml puromycin. The expression of Helios was verified with quantitative RT-PCR.

4.2.5 Generation of Bcl6 knockout DT40 B cell line

Bcl6 gene was inactivated in the DT40 B cell line with gene-targeting constructs Bcl6-bsr and Bcl6-neo to inactivate both Bcl6 alleles. These constructs were designed to disrupt the BTB/POZ domain coding regions. Selection cassettes were flanked by 1,5 kb and 1,4 kb chicken Bcl6 sequence to generate 5′ and 3′ homologous arms, respectively. The 5′ arm was generated with PCR from DT40 genomic DNA using primers C-LF (TATACTAGTCGGGAGGACCAAACTCAGCTGCCGTCCA) that creates *Spe*I site and C-LR (ATTGGATCCCGGCTTCAAAGGCGGTTGAGA-TTGAG) that generates a *Bam*HI site and a stop codon. The PCR product was digested with *Spe*I and *Bam*HI and the resulting arm was ligated to the pBluescript vector (Stratagene) in corresponding sites. The 3′ arm was generated with genomic PCR using primers C-RF (GCAGATGGAGCACGTGGTTGATACTTGCC) together with C-RR (CGCAGCTCGAGTCAGAGTACTAAGACTGGGTTTCC) that incorporates an *Xho*I site as well as a stop codon. The resulting 1,9 kb PCR product was digested with *Bam*HI and *Xho*I to get the 1,4 kb arm that was ligated into the pBluescript vector containing the 5′ arm. Finally, the selection marker cassettes where cloned into a BamHI site between the 5′ and 3′ homologous arms. The resulting constructs Bcl6-bsr and Bcl6-neo were linearized with NotI and introduced into the cells and selected for clones as described in generation of *Ikaros*$^{-/-}$ cell line with respective markers. The targeting was monitored after each transfection event with genomic PCR using primer F1 (TGCTTCTCCTGCTGCTCGATAAGGGCG) specific for the 5′ region of the chicken Bcl6 together with selection marker specific primer Bsr-F (AAACTACGA-TTGAAGAACTCATTCCACTCAAATATACCCGAAA) for Bcl6-bsr or Neo-F (TCGCCTTCTATCGCCTTCTTGACGAGTTCT) for Bcl6-neo. PCR products were probed in Southern hybridization using Bcl6 specific probe p1 (GAGGACCAAACT-CAGCTGCCGTCCAGACTT). To check successful deletion of wild type Bcl6 gene, genomic PCR product produced with primer F1 together with Bcl6 specific primer R1 (ACCCATTCTGGAGAGGCATGCTGTTCTCTG) was probed with p2 (ACCTGC-AGGCCATCAGCACTGTTTTGT) in Southern hybridization.

4.2.6 Genetic complementation of Bcl6 expression

The Bcl6 protein coding sequence was amplified from DT40 cDNA using primers Bc6-Hf (AAAAAGCTTATGGCCTCACCGGCAGACAGCTGCA) and Bc6-Nr (AAAGCTAGCTCAGCAAGCCTTGGGGAGCTCCGGA). These primers created *Hin*dIII and *Nhe*I sites that were used to clone the PCR product into corresponding site of pExpress vector (Arakawa et al., 2001) and transferred into pLoxHisD vector (Arakawa et al., 2001) as described for making Ikaros expression construct. Introduction and selection of clones were done as described for Ikaros complementation. Expression of Bcl6 was verified with quantitative RT-PCR.

4.2.7 Generation of cell line expressing Flag-tagged Bcl6

To generate affinity tagged Bcl6 expression construct, 3×Flag-tag sequence was amplified from pCMV-3Tag-1C vector (Sigma) using primers Flag-F1-Hi (TGGAAG-CTTCCACCATGGATTACAAGGAT) and Flag-R2-Hi (ATTAAGCTTTTTATC-GTCATCATCTTTGTAGTC) that generate *Hin*dIII sites. These sites were used to transfer the tag-sequence immediately 5´ to Bcl6 coding sequence in pExpress vector (Arakawa et al., 2001). The expression cassette containing chicken β-actin promoter, Flag-tagged Bcl6 coding sequence and SV40 poly-A signal was excised as *Spe*I cassette into pLoxPuro (Arakawa et al., 2001) and, after linearization, transfected into *Bcl6*$^{-/-}$ cell line with electroporation. Transfectant clones were selected in the presence of puromycin (0,5 μg/ml). Expression of 3×Flag-tagged Bcl6 was verified with quantitative RT-PCR and by western blotting with anti-Flag mAb (M2).

4.2.8 Generation of Bcl6 knockout B cell line with Pax5 expression

Pax5 expression construct (Nera et al., 2006) was transfected into *Bcl6*$^{+/-}$ cells that had first Bcl6 allele targeted with Bcl6-bsr construct. The transfected clones were selected by culturing in the presence of puromycin (0,5 μg/ml) and the expression of Pax5 in these cells was verified using quantitative RT-PCR and Western blotting. The resulting *Bcl6*$^{+/-}$/Pax5 cells were then used to inactivate the second *Bcl6* allele with Bcl6-neo as described for generation *Bcl6*$^{-/-}$ cell line.

4.3 Analysis of cell growth

To determine the growth properties of mutated cells, the cultures were diluted to 10^4 cells/ml and cultured normally. A sample of culture was taken every 24 hours until the density of living cells started to decline. The cell density of samples was measured with flow cytometer using TruCOUNT tubes (Becton Dickinson) according to manufacturer's instruction. The data is presented as a mean of two independent cultures (± SD).

4.4 Immunoprecipitation and Western blot analysis

For immunoprecipitation 2×10^7 cells were harvested and lysed in RIPA lysis buffer (150 mM NaCl; 9,1 mM Na_2HPO_4; 1,7 mM NaH_2PO_4; 1 % Nonidet P-40; 0,5 % sodium deoxycholate; 0,1 % SDS; 1 mM EDTA; pH 7.4) supplemented with 1 mM Na_3VO_4 and protease inhibitors (Complete, Roche) for 1 hour on ice. Prior the lysis, cells were either stimulated with 4 μg/ml anti-IgM mAb (M4) for indicated times or left unstimulated. The undissolved material was removed by centrifugation (10000xg for 10 minutes at 4 °C) and precleared with 20 μl of Protein A/G plus agarose particles (Santa Cruz Biotechnology). To immunoprecipitate the protein of interest, the appropriate antibody was added to the lysate, incubated 1 hour at 4 °C, 20 μl Protein A/G plus agarose particles were added and incubated over night at 4 °C. The particles were washed three times in RIPA lysis buffer with inhibitors and denatured for 10 minutes in 70 °C in the LDS sample buffer (Invitrogen) supplemented with 50 mM dithiothreitol.

For preparing whole cell lysates 1×10^6 cells were lysed with RIPA lysis buffer, centrifuged to remove undissolved material and denatured as described for immunoprecipitated samples.

Immunoprecipitated material or whole cell lysates were separated on 4–12 % bis-tris SDS-PAGE gel (Invitrogen), transferred to a nitrocellulose membrane and detected by appropriate antibodies and chemiluminescence system (ECL, Amersham or SuperSignal West Pico, Pierce).

4.5 RT-PCR and quantitative RT-PCR

RNA was isolated from 5×10^6 cells with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. 1,0 μg of RNA was used as a template to produce cDNA with 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) using oligo-p$(dT)_{15}$ primer (Roche). In RT-PCR the cDNA from 10^5 cell equivalent was amplified with primers specific for chicken Helios (Kohonen et al., 2004), Hel-f (CCTCACTGAGAATAACGAGAT) and Hel-r (CTTCTCTATAACAGCAGGTCTCT) for monitoring the expression Helios, and β-actin gene with the primers β-act-f (GTGCTGTGTTCCCATCTATCGT) and β-act-r (TGGACAATGGAGGGTCCGGATT) for a positive control.

The quantitative real-time PCR and melting curve analyses were carried out using LightCycler equipment (Roche) and FastStart DNA Master SYBR Green I kit (Roche) or with LightCycler480 equipment (Roche) and LightCycler480 SYBR Green I Master (Roche) according to manufacturer's instructions with serial dilution of template. Mg^{2+} concentration and cycling conditions were optimized for each primer pair separately. The concentration of cDNA in each sample was calculated using WT as standard with LightCycler software and normalized against the expression of GAPDH. The genes and primers used to amplify them in quantitative PCR were GAPDH (forward GAGGTGCTGCCCAGAACATCATC, reverse CCCGCATCAAAGGTGGAGGAAT), SHIP (forward (Sh-f) GGAGTCAGGACCACCTGCCACCTG, reverse (Sh-r) TCTTTCCGTGAGGCCTTGGGGTAGT), BCL6 (forward GAGAAGCCATACCCCTGTGA, reverse TGCACCTTGGTGTTTGTGAT), μS (forward GGAGAACCCCGAAAATGAGT, reverse GCCAACACCAAGGAGACATT), μM (forward GGAGAACCCCGAAAATGAGT, reverse GTTGGATGTCGTCGTCCTCT), PRDM1 (forward ACACAGCGGAGAGAGACCAT, reverse GCACAGCTTGCACTGGTAAG), PAX5 (forward GTCAGCCACGGCTGCGTCAGCAAAATAC, reverse GGCTGCTGCACCTTTGTCCGTATGAT), EBF (forward GTGGAGATCGAGCGCACCGCCTTCGTG, reverse CGTGCGTGAGCAGAACTCGGCACATTTCG), MITF (forward GGACTGTCCCTTGTTCCATCC, reverse CCGAGGTTGTCACTGAAGGTG), BACH2 (forward GCCAGTCTCTCCCCAGCTCTC, reverse GCTGGAGGTCCTCGTTCTGGT), AID (forward CCTGCGTAACAAGATGGGTTGCCATGTGGAG, reverse CGGGCAGTGAAAATGCGGAGGGTCAAGT), UNG (forward ATGGGGTTGTTTTCATGCTGTG, reverse GCAGCTCGTTTGTCTTGGAGAA), SEC24D (forward ACTGGAGGCACGCTGTACAAA, reverse ATGGCCCCGAAGAAGTCAGTA), RAB40B (forward GAGCCTACGACTTCCTGCTGA, reverse ATCTCCCTTGCCCTGATGTGT), RAB27A (forward AGAAGCGAAGCACGCAGATGAT, reverse GCACAGAGCTTCCTGACCCAGT), STX6 (forward GAGGAAGCTGACTGGTGCTGA, reverse GGTGTGAGGGCCCAATAAGAG), BRCA2 (forward ATTGGCTCTCCAAATGGATGTACGC, reverse TTCCCTTTCTTCAGCTCGGCTGTTA), RAD54B

(forward GCCAGAGTGTGGAGAGATGGTCAGA, reverse ATGTTGCCCAGAGA-AATGCTTCCAT).

4.6 Measurement of intracellular calcium

For measurements of intracellular calcium concentration in WT, *Ikaros*$^{-/-/-}$ and *Ikaros*$^{-/-/-}$ /Ik-1 cells (I), 10^7 cells were loaded with 1,5 μM Fura-2 AM (Molecular Probes) for 45 minutes at room temperature in HBSS buffer (20 mM HEPES; 118 mM NaCl; 4,6 mM KCl; 1 mM $CaCl_2$ and 10 mM glucose; pH 7.4). After loading, the cells were washed and further incubated for 20 minutes at room temperature to ensure a complete cleavage of acetoxymethyl group of the dye. After additional two washes the cells were suspended to HBSS with no $CaCl_2$ and with 0,05 mM EGTA. The fluorescence of 5×10^6 cells was monitored at 37 °C continuously with fluorescence spectrophotometer (Hitachi F2000) to see the effects of stimulation with 4 μg/ml anti-IgM (M4) antibody. The excitation wavelengths were 340 and 380 nm and emission was measured at 510 nm. The signal was calibrated by addition of 1 mM $CaCl_2$ and Triton X-100 to obtain R_{max}. Extracellular calcium was chelated with 5 mM EGTA, and pH was elevated above 8.3 by addition of Tris-base to obtain R_{min}. Calcium levels were calculated as described previously (Grynkiewicz et al., 1985) using K_d-value of 224 nM for Fura 2.

For monitoring intracellular calcium in WT, *Helios*$^{-/-}$ and *Ikaros*$^{-/-}$ cells (II), 10^6 cells were suspended in calcium buffer (PBS supplemented with 20 mM Hepes; 5 mM glucose; 0,025 % BSA; 1 mM $CaCl_2$; 0,25 mM sulfinpyrazone; pH 7.2) and loaded with 3 μM Fluo-4 AM (Molecular probes) and with 15 μM FuraRed AM (Molecular probes) for 45 minutes at room temperature. Cells were washed with calcium buffer (without Fluo-4 AM or FuraRed AM) and incubated for 30 minutes at room temperature. Intracellular Ca^{2+} levels were measured by continuous monitoring of fluorescence using flow cytometer (FacsCalibur, BD Biosciences) after addition of 2 μg/ml anti-IgM (M4) antibody. Fluo-4 and FuraRed were excited at 488 and calcium levels were recorded as emission ratio of Ca^{2+} bound Fluo-4/FuraRed (525 nm/660 nm).

4.7 Measurement of inositol phosphates

The cells were metabolically labeled by incubation for 36 hours in the presence of myo-[^{3}H]inositol (10 mCi/100 mm dish) and harvested. The cells were incubated at 37 °C for 10 minutes in HBSS, 10 minutes in HBSS containing 10 mM LiCl and then stimulated 45 seconds with anti-IgM (M4) antibody (2 μg/ml). Inositol phosphates were extracted with 10 % $HClO_4$ and separated using Amprep (SAX) mini-columns (Amersham Biosciences). Radioactivity incorporated into inositol phosphates was measured by liquid scintillation counting.

4.8 *In vitro* kinase assay

To analyze kinase activity, Lyn was immunoprecipitated as described above with following changes. Cells were lysed, immunoprecipitated and washed four times in NP40 buffer (1 % Nonidet-P40; 50 mM Tris·Cl; 150 mM NaCl; pH 8) with inhibitors (Complete protease inhibitor mix, Roche and 1 mM Na_3VO_4). The precipitates were further washed with kinase buffer (20 mM HEPES; 5 mM $Mg(CH_3COO)_2$; 5 mM $MnCl_2$; 1 mM dithiothreitol; pH 7.4). The reaction was made in kinase buffer using 2,5 μg rabbit muscle enolase as a substrate with 10 μM cold ATP and 10 μCi [γ-^{32}P]ATP

(>3000 Ci/mmol; NEN) and incubated 10 minutes at 30 °C. The reaction was terminated with addition of LDS sample buffer (Invitrogen) and boiling for 3 minutes. The samples were run in SDS-PAGE and the gel was stained, dried and developed on an autoradiography film.

4.9 Pulse-chase metabolic labeling and antibody secretion

The cells (4×10^7 cells of each culture) were harvested at culture density 0,5–1,5×10^6 cells/ml and washed with PBS twice. The cells were starved of methionine and cysteine by incubating in 1 ml methionine and cysteine free DMEM medium (Gibco) supplemented with 10 % dialyzed FCS and glutamine for 30 minutes at 40 °C, and subsequently 200 μCi [^{35}S]methionine/cysteine mix (EasyTag Express ^{35}S protein labeling mix; Perkin Elmer) was added and incubated for 15 minutes at 40 °C. The labeling was stopped by addition of 4 ml of standard culture medium supplemented with excess (5 mM) of cold L-cysteine and L-methionine (Sigma). Each cell sample was divided into five 1 ml cultures and incubated for the indicated chase times. The supernatants of cultures were carefully removed, cleared of cells by centrifugation and the supernatant was subjected to immunoprecipitation with the anti-IgM antibody (M1) as described above. After immunoprecipitation, the samples were run on 4–12 % SDS-PAGE, the gel was fixed in 15 % methanol, 7,5 % acetic acid and treated with autoradiography enhancer (Enlightning, PerkinElmer) followed by drying the gel and exposure to an autoradiography film.

4.10 Affymetrix Chicken GeneChip array analysis

Total RNA was extracted from 10^6 cells of three independent cultures of both WT and *Bcl6*$^{-/-}$ cells using TRIzol reagent (Invitrogen) according to manufacturer's instructions. The RNA was further purified with RNeasy Mini kit (Qiagen). These six purified RNA samples constitute three replicates of the WT and the *Bcl6*$^{-/-}$ cells.

Two micrograms of each total RNA sample was reverse transcribed with the One-cycle cDNA synthesis kit (Affymetrix). The double stranded cDNA preparation was purified using Sample Cleanup Module (Affymetrix) followed by *in vitro* transcription using GeneChip IVT labeling kit (Affymetrix) that incorporates biotin labeled UTP. 15 μg of labeled cRNA obtained in this way was fragmented at 94 °C for 35 minutes and then hybridized for 16 hours at 45 °C to GeneChip Chicken Genome Array (Affymetrix). After hybridization, the arrays were washed in GeneChip Fluidics Station 450 (Affymetrix) according to manufacturer's instructions.

The arrays were read with GeneChip scanner 3000 7G (Affymetrix) and GeneChip Operating Software. The .CEL files were normalized using RMA method in R software. All normalized and raw data are available at ArrayExpress (www.ebi.ac.uk/arrayexpress) under the accession number E-MEXP-2062. The genes whose expression differed in all three replicates of *Bcl6*$^{-/-}$ arrays when compared to WT arrays at least 2,5 fold ($P<0,05$) were considered.

4.11 BursaEST array analysis

Genes regulated by Ikaros were analyzed with BursaEST array (http://www.ebi.ac.uk.uk/arrayexpress, accession number A-MEXP-155). Analysis was done essentially as described (Nera et al., 2006). Briefly, mRNA from WT and *Ikaros*$^{-/-/-}$

cells was isolated using TRIzol reagent (Invitrogen) and Oligotex mRNA kit (Qiagen) and reverse transcribed in the presence of [α-^{33}P]dCTP. Labeled cDNA from WT and *Ikaros*$^{-/-}$ were hybridized on BursaEST array and visualized with phosphorimager (Fuji). Differential expression was determined using Significance Analysis of Microarrays (SAM) method with a reasonable false discovery rate (5 %). Detailed methods and data are available at Gene Expression Omnibus (GEO) and accessible through GEO series accession number GSE20946 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20946).

4.12 Chromatin immunoprecipitation

To perform chromatin immunoprecipitation (ChIP) in order to determine chromatin fragments associated with Bcl6, Pax5 and Ikaros, $2{,}0\times10^8$ of appropriate cell cultures were harvested at cell culture density $0{,}5–1{,}5\times10^6$ cells/ml. The cells were fixed for 10 minutes with 1 % formaldehyde in fresh culture medium at room temperature to fix the cells and generate protein-DNA cross-links. The formaldehyde fixation was stopped by adding glycine to final concentration of 0,3 M, incubated 5 minutes and cooled on ice. Fixed cells were washed with TBS (10 mM Tris·Cl; 150 mM NaCl; pH 7.5) and the nuclei were isolated by washing three times in MC lysis buffer (10 mM Tris·Cl; 10 mM NaCl; 3 mM $MgCl_2$; 0,5 % Igepal CA-630; pH 7.5) followed by snap freezing in liquid nitrogen. Chromatin was fragmented to an average length of 300 bp by sonication (6×1 min with 30 % duty at maximum power with Bandelin Sonopuls HD2070 and MS73 microtip) in 2 ml FA lysis buffer (50 mM HEPES; 150 mM NaCl; 1 mM EDTA; 1 % Triton X-100; 0,1 % sodium deoxycholate; 0,1 % SDS; pH 7.5) with protease inhibitors (Complete, Roche). After clearing by centrifugation, 500 µl aliquots of supernatant constituting the chromatin preparations were snap-frozen in liquid nitrogen. Aliquots were melted and diluted 1:5 with FA lysis buffer with protease inhibitors and were incubated with 100 µl Protein A/G plus agarose particles (Santa Cruz biotechnology) and centrifuged to remove non-specifically binding proteins (preclearing). To represent the input material for ChIP, 10 % of the supernatants were taken.

To precipitates chromatin fragments associated with Ikaros and Pax5, chromatin preparations from WT DT40 cells were used. To the precleared chromatin, 5 µg of anti-Ikaros (E-20) Ab and 4 µg anti-Pax5 (A-11) mAb and an equal amount of appropriate control antibodies (anti-SHIP-1 (V-19) for Ikaros and anti-IgM (M1) for Pax5 immunoprecipitation) with 100 µl of agarose particles were added and incubated for 16 hours at +4 °C. Particles were washed three times in FA lysis buffer and subsequently once in FA lysis buffer with 0,5 M NaCl, ChIP wash buffer (10 mM Tris·Cl; 0,25 mM LiCl; 1 mM EDTA; 0,5 % Igepal CA-630, 0,5 % sodium deoxycholate; pH 8.0) and TE buffer (10 mM Tris·Cl, 1 mM EDTA; pH 8.0). Finally, precipitated protein-DNA complexes were eluted with ChIP elution buffer (50 mM Tris·Cl; 10 mM EDTA; 1 % SDS; pH 7.5).

To precipitate chromatin fragments associated with Bcl6, chromatin preparations from *Bcl6*$^{-/-}$/Flag-Bcl6 cells was used for immunoprecipitation with 1,1 µg anti-3×Flag (M2) mAb and equal amount of non-specific antibody as a control (anti-IgM (M1)). Anti-3×Flag mAb was also used in an additional control immunoprecipitation from chromatin preparation of *Bcl6*$^{-/-}$/*Bcl6* cells that do not express Flag-tag. The

precipitations were done as described for Ikaros and Pax5 ChIP. However, after the precipitation, the particles from anti-3×Flag precipitation were washed 5 times with FA lysis buffer and eluted with 1 mg/ml 3×Flag peptide in TBS (30 min in 30 °C).

To release DNA from all cross-linked samples, the SDS concentration of input samples was adjusted to 0,5 % and IP, control IP and input samples were supplemented with 1,5 μg/μl pronase (Roche) and incubated 2 hours in 42 °C and 6-16 hours in 65 °C. DNA was purified using QIAquick PCR purification kit (Qiagen) and eluted to 300 μl of TE buffer.

4.13 ChIP-PCR

To analyze the Ikaros-associated DNA fragments, 5 μl of the DNA from ChIP was used as a template in 32 cycles of PCR using Phusion polymerase (Finnzymes) with GC buffer and 3,5 % of DMSO. Input samples were diluted to verify that the amplification is within a linear range. Primers SHIP-F (GTGTCATGCTCGCTCTCT-GAGCTG) and SHIP-R (ATCCATGGCTGCAGCTGGAGGAAAC) were used to detect the binding of Ikaros to *SHIP* promoter. An intronic region of *Prdm1* gene, where no Ikaros family consensus binding sequences are detected, was amplified as a negative control using primers PRDM1-F (GCCTACCACTAGGCCAGAAACCCT-TCACAT) and PRDM1-R (TACAGGCCCTGCAGTGAATAAGCCTCTTTG).

4.14 ChIP-seq

DNA from 3 replicate Bcl6, single Pax5 and 3 different control chromatin immunoprecipitation were prepared for massively parallel sequencing using sample preparation kit (Illumina) according to manufacturer's protocol. Ends of chromatin fragments of approximately 10 ng of each sample were repaired using Klenow DNA polymerase and adapter sequences were ligated to the ends of the fragments. The size of the ChIP-seq library was selected to 210–360 bp and the adapter-modified DNA fragments were amplified by PCR.

Sequencing was performed on Genome Analyzer II platform (Illumina) as single-end 36 bp reads according the manufacturer's protocols. Image analysis and base calling was done with Genome Analyzer Pipeline 1.4 software and default parameters. The resulting sequences were then aligned to chicken genome v2.1 (assembly May 2006 galGal3) using "eland extended" option of the Illumina GERALD software. Only reads that aligned uniquely to genome were used. Enrichment of sequences were detected using FindPeaks (Fejes et al., 2008) algorithm version 4.0.6 with default parameters. The output files were visualized in UCSC genome browser and exported and redrawn to make overlays of the target ChIP and appropriate control ChIP samples. Both control experiments for Bcl6 were comparable and three Bcl6 immuno-precipitations yielded similar results. For visualization purpose anti-Flag precipitation from control cell line (*Bcl6*$^{-/-}$/*Bcl6*) was overlaid with anti-Flag precipitation from Flag-Bcl6-expressing cell line (*Bcl6*$^{-/-}$/Flag-Bcl6). Pax5 immunoprecipitation from WT DT40 cells was overlaid with control antibody precipitation from the same cell line.

5 RESULTS

5.1 Ikaros regulates the activity of PLCγ2 pathway (I,II,IV)

To reveal the function of Ikaros in B cells, the *Ikaros* gene was inactivated in DT40 B cell line (I, Figure 1, IV). As a result, the cells grew more slowly and the expression of heavy and light chains of the IgM on the cell surface were increased 3,1 and 2,7 fold, respectively (I, Figure 2). The surface expression of ChB6 and CD45 remained unaltered (I, Figure 2 B), suggesting that Ikaros-deficient cells retain B cell characteristics but have a specific phenotype in BCR expression.

Given the indispensable role of Ikaros in B cell development (Georgopoulos et al., 1994; Wang et al., 1996; Kirstetter et al., 2002) and that the signal transduction through pre-BCR and BCR is essential for B cell function and development (reviewed by Kurosaki et al., 2010), we investigated whether the BCR signal is propagated normally in *Ikaros*$^{-/-}$ cells. We stimulated the BCR using anti-IgM antibody that ligates BCR molecules and activates the intracellular protein tyrosine phosphorylation cascade and eventually results in an increase in intracellular calcium concentration in DT40 cells (Takata et al., 1994).

Detection of tyrosine phosphorylated proteins before and after BCR stimulation revealed differences in phosphorylation pattern between *Ikaros*$^{-/-}$ and WT cells (I, Figure 3A). Notably, proteins of approximately 120 kDa and 85 kDa were differentially phosphorylated in *Ikaros*$^{-/-}$ cells. Immunoprecipitation with an antibody specific to B cell linker protein (BLNK, also known as SLP-65 and BASH) revealed that the tyrosine phosphorylation of BLNK (I, Figure 3 B), corresponding to the 85 kDa band observed in the phosphoblot (I, Figure 3 A), was decreased while the protein level remained the same (I, Figure 3 B). BLNK serves as a scaffolding protein that, once phosphorylated in tyrosine residues, connects several signal mediator proteins following BCR induction to propagate the BCR signals (Fu & Chan, 1997; Fu et al., 1998; Wienands et al., 1998; Hashimoto et al., 1999b; Su et al., 1999; Engels et al., 2001; Chiu et al., 2002). To understand the consequences of BLNK phosphorylation defect in *Ikaros*$^{-/-}$ B cells, we investigated the activity of Syk, a tyrosine kinase that phosphorylates BLNK (Fu et al., 1998). As the activity of Syk correlates to its own tyrosine phosphorylation (Hutchcroft et al., 1992), Syk was precipitated with a specific antibody at different time points after BCR ligation and the tyrosine phosphorylation in the immunoprecipitated samples was analyzed. The phosphorylation of Syk was impaired in *Ikaros*$^{-/-}$ cells (I, Figure 4 A). However, the protein level of Syk was also decreased after stimulation (I, Figure 4 A). The ineffective Syk function in *Ikaros*$^{-/-}$ cells may account for the reduced phosphorylation of BLNK.

Activation of phospholipase Cγ2 (PLCγ2) after BCR cross-linking requires its phosphorylation and association with BLNK (Ishiai et al., 1999a; Ishiai et al., 1999b). Too see whether the reduction of Syk and BLNK phosphorylation have any consequences on PLCγ2 we immunoprecipitated PLCγ2. As tyrosine phosphorylation of PLCγ2 correlates with its activity (Nishibe et al., 1990; Takata et al., 1994), we blotted immunoprecipitated samples with phosphotyrosine antibody. The phosphorylation of PLCγ2 was clearly decreased, while the protein was equally present in *Ikaros*$^{-/-}$ cells (I, Figure 5 A). PLCγ2 catalyzes the hydrolysis phosphatidylinositol

4,5-bisphosphate ($PI(4,5)P_2$) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). To test the activity of PLCγ2 we measured the production of inositol phosphates. The increase in inositol phosphate level after BCR stimulus was significantly ($P<0,05$) smaller in *Ikaros*$^{-/-/-}$ cells than in WT cells (I, Figure 5 B). As inositol-1,4,5-trisphosphate (IP_3) opens Ca^{2+} channels (IP_3 receptors) on endoplasmic reticulum of DT40 cells (Sugawara et al., 1997; Taylor et al., 2009), we continued to monitor the intracellular calcium levels after BCR cross-linking. Calcium mobilization was attenuated in *Ikaros*$^{-/-/-}$ cells (I, Figure 5 C; II, Figure 2 A) and was compensated with isoform Ik-1 expression in Ikaros-deficient cells (I, Figure 6 C), demonstrating a clear Ikaros-dependence for calcium mobilization. Collectively this data demonstrates that in the absence of Ikaros, the BCR signal does not propagate normally along PLCγ2 pathway (I, II, IV).

5.2 Cbl is hyperphosphorylated in *Ikaros*$^{-/-/-}$ cells and hypophosphorylated in *Helios*$^{-/-}$ cells (I-II)

In addition to the 85 kDa protein, a protein of approximately 120 kDa was differentially phosphorylated (I, Figure 3 A). Immunoprecipitation with an antibody specific to Casitas B-lineage lymphoma protein (Cbl) and blotting with phosphotyrosine antibody revealed that after Ikaros inactivation, the phosphorylation of Cbl was increased from WT levels (I, Figure 3 C). Expression of isoform Ik-1 abolished the phosphorylation indicating that Ikaros regulates the phosphorylation of Cbl (I, Figure 6 D). Cbl is a E3 ubiquitin ligase that has been shown to negatively regulate Syk by mediating its ubiquitilation after BCR stimulus (Rao et al., 2001).

Cbl is a substrate for Src family tyrosine kinase Lyn in DT40 cells (Tezuka et al., 1996). To investigate whether Lyn would account for increased Cbl phosphorylation in *Ikaros*$^{-/-/-}$ cells we analyzed the activity of Lyn *in vitro*. The tyrosine kinase activity of Lyn was increased after inactivation of Ikaros gene as evidenced by enhanced autophosphorylation and enhanced phosphorylation of enolase (I, Figure 4 B), an exogenous substrate specific for Src family kinases. Hence, the increased activity of Lyn in *Ikaros*$^{-/-/-}$ cells may cause Cbl to interfere with Syk that leads to the inefficient phosphorylation of BLNK. Thus, the combined effect of reduced Syk and BLNK phosphorylation seems to lead into inefficient activation of PLCγ2 and subsequently into a impaired increase in intracellular calcium concentration by the inositol phosphate dependent mechanism (see Figure 5).

Inactivation of Helios gene in DT40 had an opposite effect on the phosphorylation of Cbl. In *Helios*$^{-/-}$ cells Cbl was less phosphorylated than in WT cells, despite the comparable protein level (II, Figure 2 C). The reduced phosphorylation of Cbl was accompanied with increased calcium release into cytoplasm after BCR stimulus (II, Figure 2 A) suggesting that Helios has similar but an opposite function to Ikaros in regulation of PLCγ2 pathway.

5.3 Ikaros and Helios regulate the expression of *SHIP* (II)

The results suggest that the defect in the PLCγ2 pathway activation is early in the BCR signaling pathway (I). To understand how a transcription factor can regulate the signaling pathway, we searched for genes that are regulated by Ikaros. To do this, we compared the gene expression profile of *Ikaros*$^{-/-/-}$ cells to WT cells using a custom

BursaEST array. The results showed that the expression of inositol polyphosphate-5-phosphatase gene (*INPP5D*, also known as *SHIP*) is increased in *Ikaros*$^{-/-}$ cells. This finding was confirmed using quantitative PCR from Ikaros$^{-/-}$ cells as well as from cells that are complemented with Ik-1 expression. *Ikaros*$^{-/-}$ cells had approximately 2,7-fold increased expression as compared to WT and was restored close to WT level in the Ik-1-complemented cell line (II, Figure 3). Since the calcium mobilization was opposite in Ikaros and Helios -deficient B cells, we investigated whether also Helios would regulate SHIP in a similar manner. The expression of SHIP in *Helios*$^{-/-}$ cells was reduced 2,5-fold (down to 40%) from the expression level in WT cells.

Chromatin immunoprecipitation further showed that Ikaros binds close to transcription start site of *SHIP* and demonstrates that *SHIP* is a direct target of Ikaros-mediated repression (II, Figure 4). Helios can bind to the same sequences than Ikaros *in vitro* (Hahm et al., 1998) and Helios is able to activate transcription from the binding sites of Ikaros (Kelley et al., 1998). Furthermore, virtually all of the Helios proteins associate with Ikaros in the same complex (Hahm et al., 1998; Kelley et al., 1998; Sridharan & Smale, 2007). Therefore, it is likely that also Helios binds to *SHIP* promoter to regulate its expression directly and Helios seems to regulate the expression of *SHIP* in an opposite way to Ikaros.

5.4 Inactivation of *Bcl6* in B cells induces plasma cell differentiation (III)

To investigate the function of Bcl6 in B cells, a Bcl6-deficient DT40 cell line was established by targeted gene inactivation (Figure 9). These *Bcl6*$^{-/-}$ B cells grew more slowly than the wild type (WT) cells and did not reach similar cell densities (III, Figure 1A), suggesting that Bcl6 contributes to cell proliferation.

To study whether Bcl6 represses plasma cell differentiation, we first measured the levels of immunoglobulin μ heavy chain transcripts. *Bcl6*$^{-/-}$ cells expressed more secretory type (*μS*) transcripts and less transcripts encoding for membrane form (*μM*) than WT cells (III, Figure 1B). In accordance, the knockout cells had reduced surface IgM expression (III, Figure 1E). These findings suggested that *Bcl6*$^{-/-}$ cells would secrete IgM, the prominent feature of plasma cells. Pulse chase metabolic labeling confirmed that *Bcl6* inactivation induces IgM secretion into the supernatant (III, Figure 1C left panel). The secreting phenotype of *Bcl6*$^{-/-}$ cells was totally reversed by re-expression of Bcl6 in the *Bcl6*$^{-/-}$ cell line (*Bcl6*$^{-/-}$/*Bcl6* cells) demonstrating that the phenotype is specific to the loss of Bcl6 function (III, Figure 1B and 1C).

Inactivation of *Bcl6* also abolished the B cell receptor (BCR) signaling pathway. *Bcl6*$^{-/-}$ cells had no intracellular protein tyrosine phosphorylation either before or after BCR cross-linking (III, Figure 1D). Reintroduction of *Bcl6* expression to the knockout cells restored the normal pattern of protein tyrosine phosphorylation (III, Figure 1G). This indicates a role for Bcl6 in the maintenance of the BCR signaling, which is in line with findings by others (Polo et al., 2007; Ci et al., 2009; Juszczynski et al., 2009; Basso et al., 2010).

To resolve the targets of Bcl6-mediated transcriptional regulation, the expression of *Prdm1* in *Bcl6*$^{-/-}$ cells was measured first. As expected from previous findings (Reljic et al., 2000; Shaffer et al., 2000; Tunyaplin et al., 2004), a substantial expression of *Prdm1* was induced (III, Figure 2A right panel). Together these findings

demonstrate that the loss of Bcl6 in DT40 B cells induces characteristics of plasma cell phenotype.

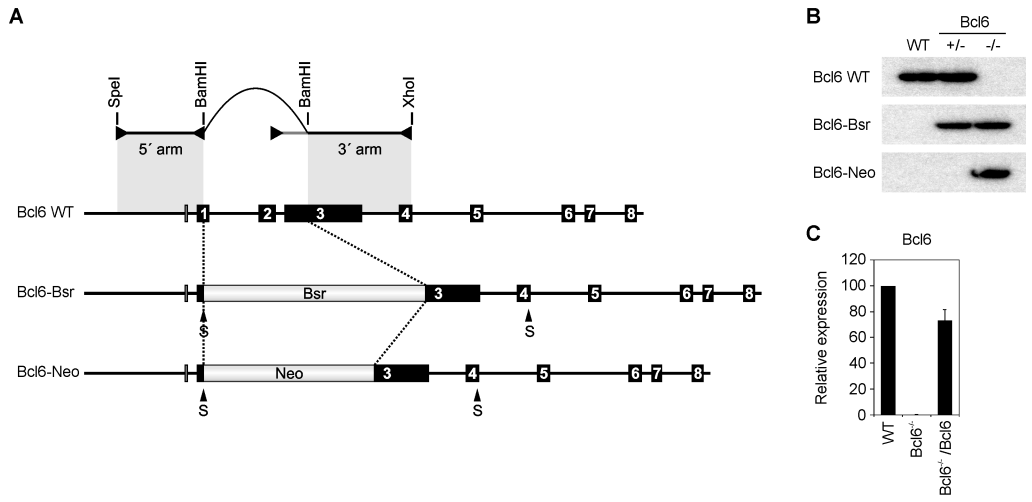


Figure 9 | Inactivation of Bcl6 gene in DT40 cell line
(A) Schematic presentation of gene targeting strategy of Bcl6 gene. The region coding for BTB/POZ domain was replaced with selectable markers (Bsr and Neo). The targeting construct was designed to introduce in-frame stop codons (arrow heads with S). (B) Correct targeting of selectable markers and loss of wild type locus as detected with Southern blotting. (C) *Bcl6* expression is lost in *Bcl6*$^{-/-}$ cells. Cells with reconstituted Bcl6 expression (*Bcl6*$^{-/-}$ /*Bcl6*) serves as a control cell line.

5.5 Switch to plasma cell gene expression program in *Bcl6*$^{-/-}$ cells (III)

To screen for other target genes of Bcl6 that may regulate plasma cell differentiation, we compared the gene expression profile of *Bcl6*$^{-/-}$ cells with WT cells using a gene expression array (Figure 10). The differential expression of select genes was confirmed by quantitative PCR (qPCR) (Figure 10 genes depicted in gray) and with *Bcl6* re-expression experiments.

Differentially expressed genes after *Bcl6* inactivation are involved in the maintenance of GC program and the repression of plasma cell program (Figure 10). Loss of *Bcl6* affected the expression of several of the genes that encode mediators or regulators of signals through BCR, genes of cell cycle regulators and genes involved in the control of DNA damage as well as genes important for B cell development and maintenance of B cell phenotype (Figure 10). Early B cell factor gene (*EBF1*) was found to be downregulated in *Bcl6*$^{-/-}$ cells and the expression of *EBF1* was normalized after *Bcl6* restoration (data not shown). Also genes involved in endoplasmic reticulum stress were upregulated in *Bcl6* knockout cells (Figure 10). These gene expression changes were accompanied by differential expression of many genes associated with protein transport and secretion such as *RAB27A*, *RAB40B* and *RHOQ* (Figure 10).

While B cell characteristics were lost, *Bcl6*$^{-/-}$ cells had also downregulated the expression of transcription factors that repress plasma cell differentiation (III, Figure 4 and Figure 10). Importantly, Bach2, a known repressor of *Prdm1*, together with *Bcl6* (Muto et al., 2004; Ochiai et al., 2006; Ochiai et al., 2008), was downregulated in *Bcl6*-

deficient cells and the number of *Bach2* transcripts was normalized after Bcl6 complementation (III, Figure 4). Also, expression of Microphthalmia-associated transcription factor gene (*MITF*), coding for a repressor of *IRF4* (Tunyaplin et al., 2004), was downregulated in *Bcl6*$^{-/-}$ cells and induced again after *Bcl6* re-expression (III, Figure 4) demonstrating a Bcl6-dependency for the expression of these factors. Thus, the loss of Bcl6 in B cells results in a dramatic change of gene expression and a shift towards a transcriptional signature of plasma cells.

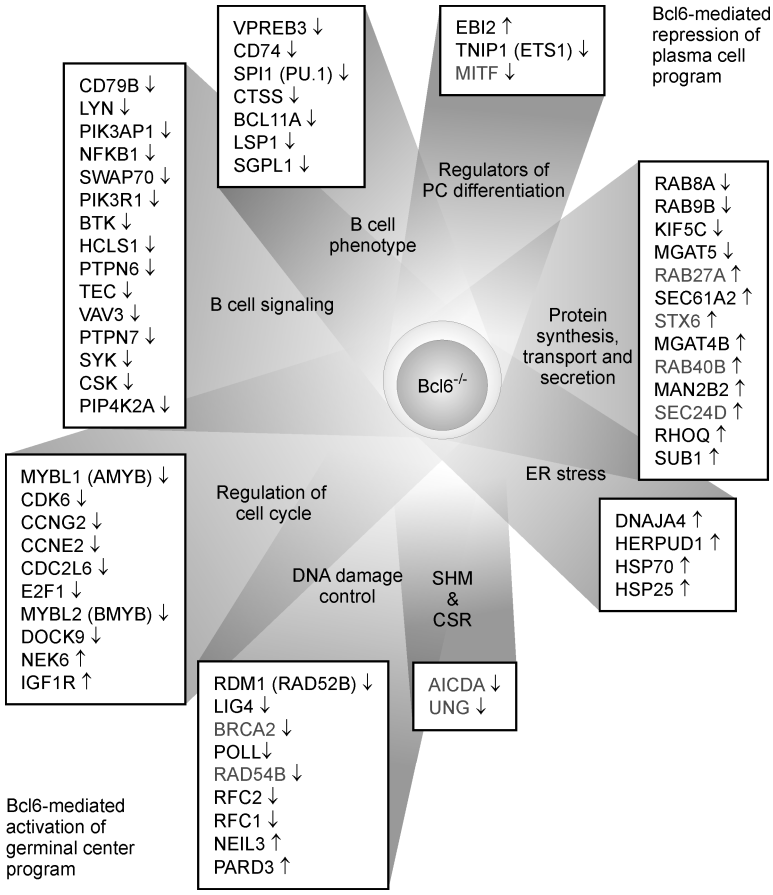


Figure 10 | Bcl6 activates germinal center program and represses plasma cell program
Selected genes that are regulated by Bcl6 based on gene expression array are organized into functional groups according to Gene Ontology terms on biological processes and literature. Inactivation of *Bcl6* resulted in downregulation of genes involved in B cell phenotype, signaling, regulation of cell cycle, DNA damage control and somatic hypermutation, as well as upregulation of genes involved protein secretion and ER stress. Also some transcription factors that repress plasma cell differentiation were downregulated. The expression changes in *Bcl6*$^{-/-}$ cells as compared to WT cells are indicated with arrows next to the gene. Genes whose expression changes were verified with qPCR are depicted in gray.

5.6 Bcl6 binds directly to genes involved in GC function and plasma cell differentiation (III)

To distinguish direct target genes of Bcl6 from those regulated through secondary Bcl6-dependent mechanisms we used Bcl6-ChIP followed by Solexa-based sequencing to identify Bcl6 binding sites in the genome wide analysis.

Only a few genes regulated by Bcl6 contained a strong Bcl6 binding site in the proximity of the transcription start site (TSS). Enriched chromatin sequences overlapped within the first non-coding exon of the *Bcl6* gene with the Bcl6 consensus binding element sequence TTCTTAGAA (Ci et al., 2009) occurring twice (III, Figure 3). Binding of Bcl6 to its own promoter suggests autoregulation, which has been reported previously (Mendez et al., 2008). Interestingly, no Bcl6 binding was observed in intronic regions of *Prdm1* or in the proximity of the gene despite its expression was upregulated in *Bcl6*$^{-/-}$ cells. As *Prdm1* and plasma cell differentiation has been suggested to be regulated by transcription factors MITF (Lin et al., 2004), IRF4 (Klein et al., 2006; Sciammas et al., 2006) and Bach2 (Ochiai et al., 2006; Ochiai et al., 2008), we specifically looked for possible direct regulation of those genes by Bcl6.

Bach2 and *MITF* were downregulated in *Bcl6*$^{-/-}$ cells and upregulated with *Bcl6* re-expression (III, Figure 4) demonstrating a Bcl6-specific effect on the expression of these genes. *Bach2* had three significant enrichment peaks within the first intron (based on the chicken genome v2.1, assembly galGal3) (III, Figure 3). As inactivation of Bcl6 downregulated the expression of *Bach2*, it is likely that binding of Bcl6 to these elements promotes the expression of *Bach2*, which contributes to the repression of *Prdm1* expression. No Bcl6-binding was observed in the gene of MITF. However, Bcl6 bound to *IRF4* gene over the region of the first ~3000 bp from the transcription start site with the peak maxima occurring in the first, the third and the fourth introns (III, Figure 3).

Also other genes expressed in germinal center were looked for Bcl6 binding. As *UNG* expression was downregulated in *Bcl6*$^{-/-}$ cells (Figure 10), Bcl6 binding to *UNG* was investigated. Bcl6 was found to bind approximately 2200 bp downstream of the TSS of *UNG* gene resulting in a peak covering the last intron of the gene (III, Figure 3). No binding was observed close to *AICDA*, the gene encoding AID.

Furthermore, Bcl6 bound to *IRF8* at approximately 3500 bp upstream and to *RHOQ* at approximately 6000 bp downstream of the TSS (Figure 11). Interestingly, Pax5 ChIP-seq data demonstrated that also Pax5 bound to *IRF8* and *RHOQ* (Figure 11). The Pax5 binding was found around and at approximately 4000 bp downstream of the TSS of *IRF8* and *RHOQ*, respectively (III, Figure 3B). The data suggests that *IRF8* and *RHOQ* genes are under the control of both Pax5 and Bcl6 through distinct sequence elements. However, in *Bcl6*$^{-/-}$ cells, where the expression of both *Bcl6* and *Pax5* is lost (III, Figure 2), the expression of *IRF8* did not significantly differ from WT cells. The expression of *RHOQ* was upregulated 10-fold in *Bcl6*$^{-/-}$ cells (Figure 10) but not significantly changed (P. Kohonen, submitted) in *Pax5*$^{-/-}$ cells (Nera et al., 2006). Bcl6 also bound to *C13orf3* gene (also known as *Ska3* in mice) (Figure 11) and seemed to be expressed in Bcl6-dependent manner.

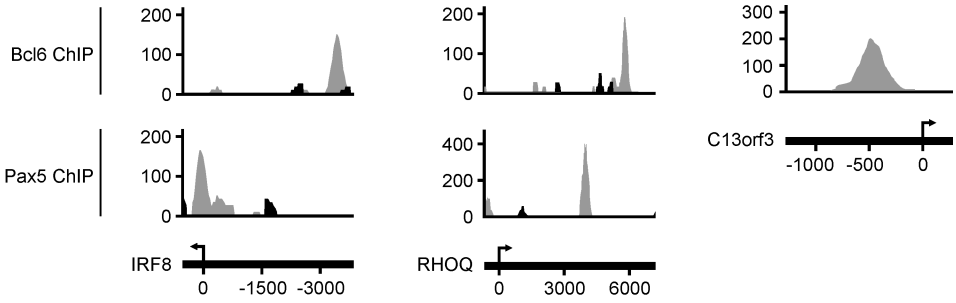


Figure 11 | Bcl6 and Pax5 binding sites in IRF8, RHOQ and C13orf3 genes
ChIP-seq experiment shows that Bcl6 immunoprecipitation-enriched chromatin fragments map to IRF8, RHOQ and C13orf3 genes, whereas Pax5 precipitation enriched fragments map closer to transcription start sites of IRF8 and RHOQ. The fragments are mapped on the positive strand of the chromosomes and the transcription start sites are indicated with an arrow. The mapped chromatin fragments from the specific immunoprecipitations are drawn in gray and fragments from negative control experiments are drawn in black.

5.7 Bcl6 represses *Prdm1* independently of Pax5 expression (III)

Based on ChIP-seq and ChIP-PCR experiments, Bcl6 doesn't seem to bind to *Prdm1* gene (data not shown), suggesting that the direct repression of *Prdm1* by Bcl6 is not the dominant mechanism for Bcl6-mediated repression of *Prdm1* (III, Figure 2). Therefore other possible indirect mechanisms were investigated. Pax5 has been shown to directly repress *Prdm1* expression (Mora-Lopez et al., 2007) and inactivation of Pax5 in B cells leads to plasmacytic differentiation (Nera et al., 2006). Pax5 protein expression was totally absent in *Bcl6*$^{-/-}$ cells and *Pax5* transcripts could not be detected (III, Figure 2). So the expression of both *Pax5* and *Bcl6* is lost with inactivation of either *Bcl6* or *Pax5* (Nera et al., 2006 and Figure 4A). Since Bcl6 and Pax5 are able to repress *Prdm1* (Tunyaplin et al., 2004; Mora-Lopez et al., 2007), it is conceivable that the deletion of either *Pax5* or *Bcl6* could relieve the repression of *Prdm1*, which in turn would downregulate the other.

To understand the hierarchy of Bcl6 and Pax5 transcription factors in the regulation of *Prdm1*, *Bcl6* was inactivated while maintaining *Pax5* expression. In order not to lose *Pax5* expression during the *Bcl6* inactivation, a DT40 mutant with a stable enforced expression of Pax5 was generated, and after that, the second *Bcl6* genomic allele was inactivated. These *Bcl6*$^{-/-}$/Pax5 cells expressed Pax5 at a similar level to wild type cells (III, Figure 2). Any phenotype observed in these cells is therefore a consequence of losing Bcl6 alone, instead of synergism of downregulated expression of both *Pax5* and *Bcl6*. Interestingly, *Prdm1* expression remained high in these *Bcl6*$^{-/-}$/*Pax5* cells (III, Figure 2), demonstrating that Pax5 is not able to repress *Prdm1* without functional Bcl6. Thus, Bcl6 does not repress *Prdm1* expression through upregulation of Pax5. Moreover, Bcl6 did repress *Prdm1* when re-expressed in *Bcl6*$^{-/-}$ cells (III, Figure 2) or in *Pax5*$^{-/-}$ cells (Nera et al., 2006).

5.8 Bcl6 is indispensable for SHM and CSR machinery (III)

Since Bcl6 directly regulates *Bach2* expression (III, Figure 3 and 4) and Bach2 is shown to be needed for *AICDA* expression, (Muto et al., 2004), we examined whether

expression of AID, a critical requirement of SHM and CSR, depends on Bcl6. The *AICDA* transcripts**,** encoding for AID, were absent in *Bcl6* knockout cells and reintroduction of *Bcl6* was capable of restoring *AICDA* expression (III, Figure 5) demonstrating that the production of AID depends on *Bcl6* expression. Since the expression of *AICDA* had also been lost in *Pax5*$^{-/-}$ cells regardless of *Bcl6* expression (Nera et al., 2006), we wanted to define more precisely the role of these factors for *AICDA* expression. Enforced *Pax5* expression in the absence of Bcl6 did not rescue *AICDA* expression, demonstrating that Pax5 cannot maintain *AICDA* expression in the absence of Bcl6 (III, Figure 5). Therefore, AID expression is dependent on the presence of both Pax5 and Bcl6.

The expression of *UNG* was also greatly reduced in Bcl6-deficient cells (III, Figure 5). UNG is responsible for uracil base removal from DNA after cytidine deamination by AID. The expression of *UNG* was upregulated after *Bcl6* re-expression, indicating the Bcl6-dependency of *UNG* expression. *Bcl6*$^{-/-}$/Pax5 cells also had elevated *UNG* expression as compared to *Bcl6*$^{-/-}$ cells (III, Figure 5). Thus, Bcl6 together with Pax5 drives the expression of both *AICDA* and *UNG*, of which UNG is directly regulated by Bcl6 (III, Figure 3), indicating a central role for Bcl6 in the regulation of SHM and CSR. Since *UNG* gene was downregulated in *Bcl6*$^{-/-}$ cells, it appears that *UNG* is a direct target gene for Bcl6-mediated activation.

5.9 DT40 B cell line provides a model to study B cell transcription factors (IV)

Use of DT40 B cell line to study the function of transcription factors regulating B cell physiology has many advantages when compared to mouse system (IV, Table 11-3). Indeed, molecular mechanisms of many aspects of B cell biology has been successfully used to study the function of B cells by loss-of-function experiments in DT40 B cell line (Buerstedde & Takeda, 2006).

Inactivation of transcription factors such as Ikaros, PU.1, EBF and Pax5 in mice have resulted in a block of B cell development (IV, Figure 11-1). As these mice do not generate B cells, the functional analysis of these factors in B cells has been problematic. DT40 B cell line has been used to circumvent these problems and several transcription factors including *PU.1*, *Pax5* and *Aiolos* that regulate development and function of B cells have been inactivated in DT40 (IV, Table 11-1). In addition to revealing novel targets in regulation of BCR signaling *Pax5*$^{-/-}$ DT40 cell lines have demonstrated that Pax5 represses plasma cell differentiation (IV, Table 11-2, Nera et al., 2006). These findings have later received support from other systems (Delogu et al., 2006; Kallies et al., 2007; Mora-Lopez et al., 2007), indicating that DT40 cell line is also valid for the analysis transcriptional regulation of plasma cell differentiation.

Ikaros family of transcription factors and Bcl6 are associated with corepressor complexes that can regulate chromatin accessibility (reviewed by Georgopoulos, 2002; Jardin et al., 2007). Several genes encoding for the subunits of these corepressors, such as *HDAC1*, *HDAC2* and *HDAC3* and their mechanisms of action have been elucidated in DT40 cell by gene inactivation (IV, Table 11-2). In addition, the molecular mechanisms of BCR signal transduction are well characterized in DT40 cells (Shinohara & Kurosaki, 2006). Therefore, DT40 B cell line is a well characterized model to study Ikaros and Bcl6 transcription factors.

6 DISCUSSION

6.1 Ikaros family transcription factors control BCR signaling

Ikaros family of transcription factors are encoded by genes, *Ikzf1* (Ikaros) (Georgopoulos et al., 1992; Hahm et al., 1994), *Ikzf2* (Helios) (Hahm et al., 1998; Kelley et al., 1998), *Ikzf3* (Aiolos) (Morgan et al., 1997), *Ikzf4* (Eos) (Honma et al., 1999) and *Ikzf5* (Daedolos) (Perdomo et al., 2000). Ikaros and Aiolos are important for B cell development and maintenance of mature and memory B cells (Georgopoulos et al., 1994; Wang et al., 1996; Wang et al., 1998; Cariappa et al., 2001), but less is known about the function of Helios in B cells (IV). The phenotype of Ikaros null mice suggests a critical B cell specific function for Ikaros, since the *Ikaros*$^{-/-}$ mice develop some T cells in adulthood but lack B cells totally (Wang et al., 1996). Ikaros is also shown to be active throughout B cell development (Tonnelle et al., 2009). However the function of Ikaros in B cells is not fully understood. The expression of Helios is highest in T cells (Hahm et al., 1998; Kelley et al., 1998), but it is also found in B cell progenitors, some B cells and B cell organs such as bursa of Fabricius (Mustonen et al., 2010) and GCs (Kohonen et al., 2004; Papathanasiou et al., 2009).

In this work, a novel function for Ikaros and Helios in B cells is established (I-II), by using DT40 B cells as a model system (IV). Both Ikaros and Helios are involved in the regulation of BCR signaling. This work also shows that Ikaros and Helios have opposite functions. Inactivation of Ikaros causes reduced response to BCR stimulus in terms of PLCγ2 pathway activity that leads to reduced release of calcium from intracellular stores (I). This finding demonstrates that Ikaros is needed for efficient signaling through BCR. Previously, similar role has been suggested to Aiolos in setting a threshold for B cell activation via BCR (Wang et al., 1998; Cariappa et al., 2001). *Helios*$^{-/-}$ cells had a stronger calcium response than observed in wild type cells demonstrating that also Helios regulates the strength of calcium response after BCR engagement (II). Interestingly, the inactivation of Helios suppressed BCR calcium response indicating a role objecting Ikaros in the regulation of BCR signaling.

Signaling through BCR regulates early B cell development (Kitamura et al., 1991), and enables survival during immature (Meffre & Nussenzweig, 2002) and mature stages (Kraus et al., 2004). Magnitude and duration of BCR signal determine cell fate decisions during the B cell development (Casola et al., 2004; Kurosaki et al., 2010). The transition of immature B cells from bone marrow to spleen is impaired in mice with mutations that impair BCR signaling (Torres et al., 1996; Turner et al., 1997). The requirement of Ikaros for efficient BCR signal transduction gives a prediction that in the absence of Ikaros, the passing through developmental checkpoints would be compromised. Indeed, mice engineered to express reduced amounts of Ikaros ($Ik^{L/L}$) (Kirstetter et al., 2002) have impaired development into mature B cells resulting into a reduced transition of immature B cells from bone marrow to spleen. Similarly, the expression of the dominant negative isoform (Ik-6) in cells committed to the B lineage impairs the development into mature B cells (Tonnelle et al., 2009). Therefore, the results with *Ikaros*$^{-/-}$ DT40 B cells suggest that the inability of $Ik^{L/L}$ immature B cells to effectively pass transitional stage and fully mature is due to reduced function of Ikaros to maintain BCR signaling. Thus, the findings presented here provide a

molecular explanation for the need of Ikaros throughout the later stages of the B cell development and maintenance of B cells (I, II, IV). Together with the data from Ikaros-deficient mice the data with *Ikaros*$^{-/-}$ DT40 B cells suggests that Ikaros regulates the function of B cells by sensitizing them to developmental checkpoints. Given the opposing function of Helios to Ikaros, the concerted action of Helios and Ikaros balances the outcome in these checkpoints.

The idea of Ikaros family members acting in the opposite manners to each other in the regulation of gene expression is a novel concept. Ikaros family members are previously reported to rather have a parallel function in the regulation of gene expression during the B cell development. The repressing function of Ikaros competes with the activating function of EBF1 at the pro-B stage to balance the expression of λ5 (Sabbattini et al., 2001; Thompson et al., 2007; Ma et al., 2010). λ5 drives the proliferative expansion via pre-BCR signaling. A sudden increase in Aiolos expression is observed at the small pre-B stage, when pre-BCR is downregulated (Thompson et al., 2007). The increase in Aiolos expression coincides with the downregulation of λ5 expression and the λ5 protein is not fully downregulated in the absence of Aiolos (Thompson et al., 2007), despite the continuous expression of Ikaros. The upregulated expression of Aiolos at the small pre-B stage would therefore reinforce the effect of Ikaros in repressing λ5 gene expression. Accordingly, they bind to same DNA sequences and are involved in the same repression complexes (Morgan et al., 1997; Hahm et al., 1998; Kelley et al., 1998). The parallel function of Ikaros and Aiolos is further emphasized by the finding that both Ikaros and Aiolos repress c-Myc expression in pre-B cells to shut down the pre-B cell expansion (Ma et al., 2010). As this idea provides a model for developmental stage-specific regulation of common target genes by Ikaros and Aiolos, the biological consequences of counter-regulatory effect of Helios and Ikaros are not yet understood. However, the findings that Ikaros represses class switching to IgG_{2b} (Sellars et al., 2009), whereas Helios transgenic mice produce increased amount of IgG_{2b} (Dovat et al., 2005) may reflect the biological outcome of the counter-regulatory function between Ikaros and Helios.

6.2 Direct regulation of *SHIP* by Ikaros and Helios

To establish a connection between the transcription factors Ikaros and Helios in the nucleus with the BCR signaling pathway initiating at the cell surface, a global gene expression change induced by inactivation of *Ikaros* was analyzed. One interesting target gene, whose expression change was verified with quantitative PCR was *SHIP* encoding for SH2-containing inositol phosphatase (SHIP). SHIP dephosphorylates 5-position of the inositol ring of phosphatidylinositol-3,4,5-trisphosphate $PI(3,4,5)P_3$ (Figure 12, Damen et al., 1996). Both Ikaros and Helios were found to regulate its expression in the opposite way, in accordance to the regulation of BCR signaling. Ikaros represses *SHIP* directly by binding close to *SHIP* transcription start site. Despite the fact that it was not directly shown that Helios binds to *SHIP* gene, there are several lines of evidence that strongly suggests that it does. Firstly, Ikaros and Helios are highly conserved, especially in the DNA-binding domain (Kohonen et al., 2004; John et al., 2009). Also Aiolos and Ikaros have comparable similarity in DNA-binding domain and they bind to the same DNA sequences (Morgan et al., 1997; Liippo et al., 1999). Therefore, the consensus binding sequences are likely to be identical between

Ikaros, Helios and Aiolos. Secondly, Ikaros family members are associated in the same macromolecular complexes (Hahm et al., 1998; Kelley et al., 1998; Kim et al., 1999; Koipally et al., 1999; Sridharan & Smale, 2007) and colocalize in nuclei (Hahm et al., 1998; Kelley et al., 1998). Furthermore, Helios is shown to dimerize with Ikaros (Hahm et al., 1998; Kelley et al., 1998) and to quantitatively associate with Ikaros complexes with virtually all Helios proteins being associated with Ikaros (Hahm et al., 1998). Thirdly, Helios can directly activate transcription from Ikaros DNA-binding sequences (Kelley et al., 1998). Therefore, *SHIP* gene is very likely to be bound also by Helios and to be a direct target of Helios-mediated activation.

SHIP is an important negative regulator of BCR-induced signals (Liu et al., 1998; Okada et al., 1998; Hashimoto et al., 1999a). Correct regulation of plasma membrane phospholipids (Figure 12) in the proximity of BCR is important for keeping the initiation, duration, magnitude and attenuation of the signals balanced (Kurosaki, 2002; Leung et al., 2009). Localization of SHIP to membrane leads to dephosphorylation of phosphatidylinositol-(3,4,5)-trisphosphate $PI(3,4,5)P_3$ to phosphatidylinositol-(3,4)-bisphosphate $PI(3,4)P_2$. Studies in DT40 cells indicate that SHIP is a critical inhibitor of membrane recruitment of Btk (Bolland et al., 1998). Inactivation of SHIP results in Btk membrane association and hyperresponsive BCR signaling (Bolland et al., 1998). Enforced membrane association of Btk can bypass the BCR signaling deficiency in conditions where $PI(3,4,5)P_3$ levels are reduced, e.g. with constitutively membrane associated SHIP (Bolland et al., 1998). As membrane recruitment and activation of Btk is mediated by $PI(3,4,5)P_3$ but not $PI(3,4)P_2$ (Salim et al., 1996; Rameh et al., 1997), SHIP activity negatively regulates Btk (Bolland et al., 1998; Scharenberg et al., 1998). Btk activates PLCγ2 in response to BCR ligation (Takata & Kurosaki, 1996). Also PLCγ2 contains a PH domain that mediates interaction with $PI(3,4,5)P_3$, suggesting that maintaining $PI(3,4,5)P_3$ levels is important to PLCγ2 membrane localization. However, mutations that prevent the BLNK-PLCγ2 interaction almost totally prevent the membrane localization and activation of PLCγ2 suggesting instead, that the primary mechanism employed by PLCγ2 for membrane recruitment is through phosphorylated BLNK (Ishiai et al., 1999a; Ishiai et al., 1999b; Chiu et al., 2002). Therefore, it seems that increased SHIP expression in *Ikaros*$^{-/-}$ cells results into an inefficient membrane assembly and activation of BCR signaling complex that is needed for PLCγ2 activation, IP_3 generation and subsequent calcium release after BCR stimulus. Accordingly, *Helios*$^{-/-}$ DT40 cells have increased calcium response, exactly as observed in *SHIP*$^{-/-}$ DT40 cells (Okada et al., 1998). Therefore, the altered regulation of SHIP at least partly explains the reduced activation of PLCγ2, inositol phosphate production and release of calcium in *Ikaros*$^{-/-}$ DT40 cells and the opposite phenotype in *Helios*$^{-/-}$ DT40 cells.

The regulation of SHIP by Ikaros and Helios has also implications on the functional level. As expected based on its role in the regulation BCR signaling, SHIP is also needed for normal B cell development and activation (Liu et al., 1998; Helgason et al., 2000; Nakamura et al., 2004). *SHIP*$^{-/-}$ mice have reduced B cell populations after the pre-B cell stage and increased number of IgM^+IgD^+ mature B cells in the spleen (Helgason et al., 2000). Conditional deletion of SHIP in B cells, however, points to a phenotype at the germinal center stage, where SHIP plays an essential role in the regulation of high affinity B cell clones in T-dependent responses (Leung et al., 2009).

SHIP$^{-/-}$ GC B cells have normal somatic hypermutation but the positive selection for high affinity clones is impaired (Leung et al., 2009). *SHIP*$^{-/-}$ mice generate germinal centers spontaneously and have increased rate of isotype switching (Liu et al., 1998; Helgason et al., 2000).

Helios is expressed in B cells at a low level (Kohonen et al., 2004; Papathanasiou et al., 2009), although it has been suggested that downregulation of Helios is required for normal B cell function (Dovat et al., 2005) and the expression of Helios is downregulated during the differentiation into mature B cells (Papathanasiou et al., 2009). The counter-regulatory functions of Helios to Ikaros in B cells may therefore serve to fine-tune the function of Ikaros and other family members. Interestingly, the Helios transgenic mice have normal numbers of B cells and Helios seems to affect B cell function in terms of directing class-switch recombination to IgG_{2b} class and prolonging the cell survival (Dovat et al., 2005).

The exact mechanism by which Ikaros and Helios exert their opposite regulatory function remains unknown and more experiments are required to address this question. However, this is the first demonstration that Ikaros family members regulate a common target gene in the opposite way. It would be interesting to find out which part of Helios protein mediates this function, while existing in the complexes. As Ikaros has been shown to be able to both activate and repress transcription (Koipally & Georgopoulos, 2000; Georgopoulos, 2002; Koipally & Georgopoulos, 2002; Koipally et al., 2002), investigation of preferential association of Helios with activating chromatin modifiers may provide clues. It would also be interesting to further delineate the concept of counter regulation of Ikaros by Helios in hematopoietic stem cells and T cells where Helios is more abundantly expressed (Papathanasiou et al., 2009). The signaling through TCR is analogous to BCR signaling, employing PH-domain containing kinases (the membrane docking site is catabolized by SHIP) and PLCγ (reviewed by Smith-Garvin et al., 2009). Ikaros might have a similar role in T cells as a regulator of the antigen receptor signaling pathway as demonstrated in this work for B cell antigen receptor (Avitahl et al., 1999). Given the counter-regulatory function in B cells, it would be especially interesting to find out the function of Helios in T cells. Indeed, both SHIP and Helios have recently been proposed to function in regulatory T cell development (Kashiwada et al., 2006; Getnet et al., 2010; Thornton et al., 2010). However, Helios seems to be dispensable for αβ and γδ T cells, NKT cells, and regulatory T cells (Cai et al., 2009).

Previous work show that unbalanced expression of Ikaros family members may induce lymphomas, as *Ikaros*$^{+/-}$ mice as well as mice heterozygous for dominant negative mutation in Ikaros gene develop severe T cell lymphoma (Winandy et al., 1995). The strength of the signal through antigen receptor is decisive in cell fate choices in T cells as well. In line of these findings Ikaros is suggested to set a threshold for T cell activation (Avitahl et al., 1999). Accordingly, aberrant signaling can lead to development of autoimmune diseases and leukemia (Refaeli et al., 2008; Young et al., 2008; Zikherman & Weiss, 2009). Thus, balanced expression of Ikaros and Helios may contribute to preventing the onset of these diseases.

A function of Ikaros has been shown also in the Ig class-switch recombination. Studies with $Ik^{L/L}$ mice (Kirstetter et al., 2002) have revealed that Ikaros inhibits accessibility of AID to the *γ2b* and *γ2a* genes by suppressing active chromatin marks,

and Ikaros interacts directly with isotype gene promoters as well as regulates IgH 3´ enhancer (Sellars et al., 2009). The regulation of IgH locus accessibility has been reported also with *Ikaros*$^{-/-}$ mice (Reynaud et al., 2008). Thus, Ikaros promotes class switching to other classes in the expense of switching to IgG_{2a} and IgG_{2b}. In accordance to the counter-regulatory role for Helios and Ikaros, the transgenic Helios expression in B cells appears to promote class switching specifically to IgG_{2b} (Dovat et al., 2005). This suggests another stage-specific physiological function for Ikaros family members, as Helios is abundantly expressed in germinal centers (Kohonen et al., 2004). However, the mechanism and target genes of Helios involved in this process are not yet known. Also Aiolos is demonstrated to have a function in the B cell selection in the germinal centers and Aiolos is required for generation of high-affinity plasma cell population and long term memory in a B cell intrinsic manner (Cortes & Georgopoulos, 2004).

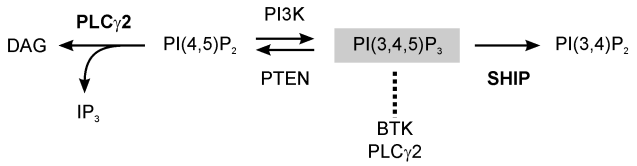


Figure 12 | SHIP dephosphorylates PI(3,4,5)P_3 in plasma membrane
PI(4,5)P_2 in phosphorylated by PI3K to yield PI(3,4,5)P_3 that can recruit PH-domain containing proteins such as Btk and PLCγ2. PI(3,4,5)P_3 is a substrate for dephosphorylation by SHIP that yields PI(3,4)P_2. PLCγ2 cleaves PI(3,4,5)P_3 into IP_3 and DAG, which resides in the membrane. IP_3 dissolves into cytoplasm and is bound by the IP_3 receptors on the endoplasmic reticulum surface.

6.3 Target genes and function of Bcl6

Bcl6 is considered the master regulator of germinal centers, since inactivation of Bcl6 in mice results in total lack of germinal centers and Bcl6 regulates several functions in GCs. Bcl6 is the key repressor of plasma cell differentiation. The inactivation of Bcl6 in DT40 B cells induced spontaneous acquisition of several plasma cell characteristics, including shift from B cell gene expression program to that of plasma cells and secretion of IgM antibody (III).

In effort to find novel direct target genes for Bcl6-mediated regulation, an analysis combining global Bcl6 binding sites to gene expression changes after Bcl6 gene inactivation was done. Several studies, including this work, have demonstrated that Bcl6 represses the expression of *Prdm1* (Shaffer et al., 2000; Tunyaplin et al., 2004), the gene encoding Blimp-1. Surprisingly, no binding of Bcl6 was observed in *Prdm1*, unlike suggested by previous reports (Tunyaplin et al., 2004; Ochiai et al., 2008). This does not demonstrate that Bcl6 cannot bind to *Prdm1*, but suggests that direct binding is not the only or prevailing mechanism for Bcl6-mediated repression of *Prdm1*. Accordingly, another study elucidating global binding sites of Bcl6 in mouse cells was also unable to find *Prdm1* among the core target genes (Basso et al., 2010). Instead of *Prdm1*, Bcl6 bound to *Bach2* gene and, accordingly, the expression of *Bach2* in *Bcl6*$^{-/-}$ DT40 B cells was dramatically downregulated. Bach2 is shown to repress *Prdm1*

directly (Ochiai et al., 2006; Ochiai et al., 2008). These findings with Bcl6-deficient cells have two important implications: (i) Bcl6 may primarily suppress plasma cell differentiation and *Prdm1* expression via indirect routes and (ii) Bcl6 can activate gene expression.

The secretory pathway is ubiquitous to all cells and essential for Ig secretion. Resting B cells have little ER and have only surface Ig. After activation and during plasma cell differentiation surface Ig is switched to secreted type by alternative splicing of the transcript. To deal with the heavy secretory loads, plasma cells expand their ER network containing chaperones to correctly fold the Ig molecules. The proteins are packed into vesicles delivering them into Golgi for glycosylation and for transport to plasma membrane in secretory vesicles. Bcl6 repressed several G proteins that regulate secretory vesicle traffic. As no binding to these were observed, they are likely to be secondary targets, presumably through Xbp1, that is one of the key transducers of the B cell unfolded protein response. Interestingly Bcl6 repressed the expression of *RHOQ* and bound to its promoter. RhoQ (also known as TC10) is a G protein that is part of the Exo70 exocyst complex (Inoue et al., 2003) involved in vesicle trafficking and membrane fusion. The direct binding of Bcl6 to *RHOQ* suggests that Bcl6 has also a role in repressing secretion directly.

Interestingly, *EBI2* (also known as *GPR183*) is upregulated when Bcl6 is inactivated in B cells. As EBI2 expression together with Blimp-1 drives the extrafollicular plasma cell differentiation (Gatto et al., 2009; Pereira et al., 2009), the Bcl6 expression may drive germinal center fate by downregulating EBI2 expression and extrafollicular plasma cell differentiation. Bcl6 also bound to the gene *C13orf3* (also known as *Ska3*), a recently identified regulator of cell division (Daum et al., 2009; Gaitanos et al., 2009; Theis et al., 2009). Binding of Bcl6 to *C13orf3* is also supported by recent findings of others in human primary GC B cells (Ci et al., 2009). This demonstrates another direct link to regulation of centrocyte proliferation.

The fact that the expression of so many genes was affected without an identifiable Bcl6 binding may reflect the situation where the dramatic gene expression signature associated with cellular differentiation process has settled to an equilibrium and therefore do not represent the Bcl6 targets but instead those of Blimp-1 or Xbp1 for example. In fact, several identified genes involved in secretion and ER stress are known Xbp1 targets (Shaffer et al., 2004). Alternatively, Bcl6 may be recruited to some of the genes regulated, but not bound by Bcl6 in the present experiment, through a mechanism involving another sequence specific transcription factor, similarly as demonstrated for Miz-1 mediated recruitment of Bcl6 to *CDKN1A* promoter (Phan et al., 2005).

Bcl6 was found to bind to IRF4 but the expression of IRF4 was not significantly affected by Bcl6 inactivation. This may be due to other factors regulating IRF4 expression that potentially can override the effects of Bcl6, such as NF-κB (Saito et al., 2007).

6.4 DNA damage control, SHM and CSR

The expression of Bcl6 in germinal centers occurs concomitantly with the expression of AID and the SHM and CSR, the processes where AID is pivotal. The data presented in this work show that Bcl6 is required for the expression of AID and UNG. This

suggests that Bcl6 contributes to germinal center phenotype through AID. AID is indispensable for initiation of SHM, CSR and gene conversion (Muramatsu et al., 2000; Revy et al., 2000; Okazaki et al., 2002; Yoshikawa et al., 2002). Furthermore, as Bcl6 is specifically expressed in GC B cells, it seems to account for the GC-specific expression of AID. Recently, AID has been shown to affect the size of the germinal centers by regulating B cell susceptibility to apoptosis (Zaheen et al., 2009), providing another mechanism by which Bcl6 could control GCs. This finding also has implication to lymphoma formation, as the aberrant activity of both Bcl6 and AID are linked to germinal center-derived B cell lymphomagenesis (Klein & Dalla-Favera, 2008; Pasqualucci et al., 2008). Interestingly, SHM is shown to occur also on Bcl6 gene (Pasqualucci et al., 1998).

AID is highly potent in inducing mutations. To prevent potentially harmful effects, the mutations must be confined to Ig loci. The protection is achieved at least on three levels. Firstly, the expression of AID is temporally restricted to activated B cells within GCs (Muramatsu et al., 1999) where the redundant mutations can be eliminated. Secondly, deamination is targeted only to actively expressed loci such as Ig loci and to a lower extent to other loci such as Bcl6 (Betz et al., 1994; Pasqualucci et al., 1998; Shen et al., 1998; Inlay et al., 2006). Thirdly, the used repair mechanisms differ by locus in question. AID-induced lesions at the Ig loci are predominantly resolved by mutagenic mechanisms, whereas non-Ig loci are resolved by high-fidelity mechanisms (Liu et al., 2008).

Previous work has suggested that Bcl6 controls the extent of DNA damage in germinal centers. Bcl6 represses genes that are involved in the pathway detecting DNA damage (Ranuncolo et al., 2008). On the other hand the function of Bcl6 itself is suppressed by extensive DNA damage through degradation (Phan et al., 2007). The findings presented here ads to this rheostat function of Bcl6. Promotion of AID and UNG gene expression by Bcl6 shows that Bcl6 is involved in the induction of the damages in the first place. Thus, the role of Bcl6 in the regulation of AID and UNG by Bcl6 closes the cycle in the regulation AID-induced mutagenesis during SHM and CSR within germinal centers (Figure 13) and provides an explanation for temporal regulation of AID and UNG. The regulation of the mutagenic cycle is also connected to the regulation of cell cycle. Therefore, rapid removal by proteasomal degradation of Bcl6 in response to signal from high affinity BCR, provides a physiological checkpoint that prevents non-beneficial and potentially harmful mutagenesis when it is not needed. Therefore, Bcl6 seems to constitute a nodal point that connects several key functions of germinal centers through a single factor.

Given the central role of Bcl6 in the regulation of mutagenesis, it is not surprising that the function of Bcl6 is tightly regulated at several levels including transcriptional control as well as with post-translational mechanisms. Chromosomal translocations of GC non-Hodgkin lymphomas often involve breakpoints in switch region or the SHM target regions of immunoglobulin locus (Kuppers, 2005). Indeed, in the lack of regulation, Bcl6 has proven to be hazardous. The 5′ non-coding region of Bcl6 is the target of translocations, deletions and somatic point mutations. A translocation is observed in 20–40 % of diffuse large B cell lymphoma, 15 % of follicular lymphoma and many other B cell lymphomas (Bastard et al., 1994; Offit et al., 1994; Jardin et al., 2002) and in addition to translocation to Ig locus, several other partners have been identified (Jardin et al., 2007). Also deletions of different sizes can be associated with

translocations but they also occur independently in Bcl6 locus (Nakamura et al., 1996; Bernardin et al., 1997). Somatic mutation is actively targeted to Bcl6 5´ region and in addition to having association to lymphomas (Migliazza et al., 1995) can occur normally (Pasqualucci et al., 1998; Shen et al., 1998). As AID is important in development of lymphoma (Mahowald et al., 2008; Pasqualucci et al., 2008; Robbiani et al., 2008; Tsai et al., 2008), the data with *Bcl6*$^{-/-}$ cells suggest that AID is at least partly responsible for malignant nature of deregulated Bcl6. If this was true, expression of AID in Bcl6-deficient cells should be able to promote lymphomagenesis, or *vice versa*, inactivation of AID in Bcl6-dependent lymphoma cells should abrogate lymphoma. The latter is shown to be true with a model of germinal center derived B cell non-Hodgkin's lymphoma (Pasqualucci et al., 2008). Therefore, the first direct evidence shown here of Bcl6-dependent expression of AID and UNG, that are both involved in the same mutagenic pathway, gives a clear explanation of oncogenic activity of Bcl6.

IRF4 is shown to physically interact with Bcl6 (Gupta et al., 1999) and to promote CSR (Klein et al., 2006), suggesting a collaborative function in CSR. Indeed, IRF4 promotes the expression of AID (Sciammas et al., 2006; Luo & Tian, 2010). The requirement for CD40 signaling for CSR (reviewed by Kracker et al., 2010) suggests that T cell help in the light zone after affinity maturation starts class-switch recombination. The few germinal center cells that express IRF4 also express Blimp-1 but not Bcl6 (Falini et al., 2000; Cattoretti et al., 2006). As Pax5 is also needed for AID expression, the upregulated IRF4 expression by T cell help would take over the role of Pax5 and Bcl6 in maintaining AID expression (III and Nera et al., 2006).

Finally, the data presented in this work show that Bcl6 directly promotes the expression of Bach2 and UNG that are important regulators of plasma cell differentiation and somatic hypermutation/class switching, respectively, the central functions of germinal center B cells.

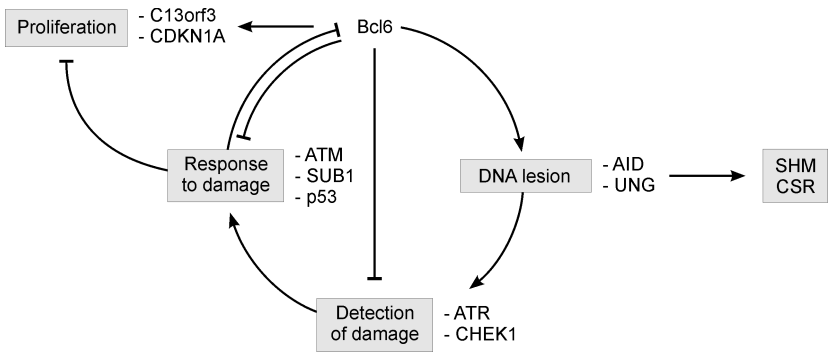


Figure 13 | A proposed model for the involvement of Bcl6 in the control of SHM and CSR
Bcl6 promotes somatic hypermutation (SHM) and class-switch recombination (CSR) by activating the expression of AID and UNG. Meanwhile Bcl6 allows the mutations and proliferation to occur in germinal centers by suppressing detection of and response to DNA damage as well as by promoting cell cycle by regulating key genes involved in the processes. The excessive DNA damage can also lead to the degradation of Bcl6 protein.

6.5 A model for the induction of plasma cell differentiation

These findings support a scheme that Bcl6 provides a triggering point for a rapid induction of plasma cell differentiation in germinal centers. The signals and mechanisms that induce plasma cell differentiation after germinal center response are not comprehensively understood. However, a signal through high-affinity BCR is required (Phan et al., 2006), which may provide the physiological trigger. After BCR ligation, Bcl6 is phosphorylated by ERK2, which results in the degradation of Bcl6 protein via ubiquitin/proteasome pathway (Niu et al., 1998). In addition, the repressive capacity of Bcl6 is also inhibited by acetylation that impairs the interaction of Bcl6 with histone deacetylases (Bereshchenko et al., 2002). Losing the repressive action of Bcl6 would alleviate the repression of Blimp-1 (Shaffer et al., 2000; Tunyaplin et al., 2004). Centrocytes also receive different signals from other light zone lymphocytes. CD40 signaling has been demonstrated to induce NF-κB-mediated upregulation of IRF4 in B cells (Saito et al., 2007). IRF4 is a transcription factor that is required for plasma cell development in a B cell intrinsic fashion (Klein et al., 2006; Sciammas et al., 2006). IRF4 is capable of inhibiting Bcl6 gene expression by binding to sequences flanking Bcl6 transcription start site (Saito et al., 2007). CD40 signaling is also reported to disrupt the interactions of Bcl6 with its corepressors (Polo et al., 2008). Thus, the combined action of CD40 and BCR signals in the light zone centroblasts seems to converge at Bcl6 and shut down Bcl6 function both in the protein and transcription level to extinguish germinal center phenotype and to allow subsequent development.

The finding that Bcl6 directly upregulates Bach2 provides a new route for Bcl6-mediated repression of Blimp-1. Bach2 is expressed in B lineage from early B cell progenitors to mature B cell stage but is absent in plasma cells (Muto et al., 1998). *Prdm1* is upregulated in Bach2-deficient B cells (Muto et al., 2004). Bach2 represses *Prdm1* expression by binding to its promoter (Ochiai et al., 2006) and, together with Bcl6, to an intronic enhancer region of *Prdm1* (Ochiai et al., 2008). Bach2-deficient mice have a defect in SHM and CSR, and Bach2 is not required for plasma cell differentiation (Muto et al., 2004).

Inactivation of Bcl6 also reduced the expression of Microphthalmia-associated transcription factor gene *MITF* (III). *MITF* is expressed in many cell types. The lymphoid isoform (MITF-L) is expressed in resting B cells and is downregulated by activating signals (Lin et al., 2004). MITF suppresses plasma cell differentiation. Defective MITF activity results in B cell activation and immunoglobulin secretion, whereas ectopic MITF expression suppressed plasma cell development and IRF4 expression (Lin et al., 2004). However, the nature of MITF-mediated repression of IRF4 is currently not understood. IRF4 promoter contains potential MITF binding sites but does not seem to bind to or repress IRF4 directly *in vitro* (Lin et al., 2004), suggesting that other factors are involved in mediating DNA binding of MITF. This work therefore suggests that Bcl6 may repress plasma cell differentiation and *Prdm1* also via MITF-mediated repression of IRF4. However, the mechanism by which Bcl6 affects MITF expression may not be direct, as no significant binding of Bcl6 to MITF was observed.

6.6 Emerging roles for Bcl6

A recent study has extended our understanding of Bcl6 by showing a function for Bcl6 in the pre-B cell stage (Duy et al., 2010). In addition to its role in suppression of DNA damage induced apoptosis in germinal centers (Klein & Dalla-Favera, 2008), Bcl6 seems to have a similar function during immunoglobulin light chain gene rearrangement (Duy et al., 2010). IL-7-Stat5 signaling in large cycling pre-B cells actively prevents Ig light chain gene recombination (Malin et al., 2010). Phosphorylation of Stat5 at Y694, leads into a suppression of Bcl6 (Walker et al., 2007; Duy et al., 2010). The level of DNA damage is low at this stage, as the Ig light chain genes are still at the germline configuration. Pre-BCR signaling downregulates IL-7Rα, reduces IL-7 responsiveness and leads to Stat5 dephosphorylation and in an activation of Bcl6 transcription (Schebesta et al., 2002; Johnson et al., 2008; Nakayama et al., 2009). Pre-BCR signaling also induces IRF4 as well as IRF8 and thereby IgL gene rearrangement (Schebesta et al., 2002; Johnson et al., 2008). The recombination involves DNA double stranded breaks and induces DNA damage response genes such as *CDKN1A*, *CDKN1B* and *ARF*, that are all repressed by Bcl6 (Duy et al., 2010). Pre-B cell stage therefore includes analogical coupling of B cell proliferation and DNA damage tolerance to GCs and highlights the central role for Bcl6 in generation of diverse B cell repertoire in various stages of B cell development.

Recent data indicate that Bcl6 is an important regulator of follicular helper T (T_{FH}) cell development and function in germinal centers (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009; Poholek et al., 2010). Blimp-1 also antagonizes Bcl6 in the T_{FH} cell differentiation (Johnston et al., 2009) suggesting that similar Bcl6-Blimp-1 mutual counter-regulation as in B cells to plasma cell transition is also involved in T cells. As CD4+ T cell help is needed for generation of germinal centers, memory B cells and long-lived plasma cells, T_{FH} represents the T cell subset specialized for B cell help in germinal centers (reviewed by McHeyzer-Williams et al., 2009). Also the Bcl6-Blimp-1 axis seems to be active in bone cells (Miyauchi et al., 2010). As Bach2 also has a demonstrated function in some $CD4^+$ T cells (Lesniewski et al., 2008), the finding that Bcl6 activates Bach2 expression may also have implications in T cells. However, direct experiments are needed to address this question.

7 CONCLUDING REMARKS

The contribution of an individual transcription factor for the phenotype is generally investigated using loss-of-function approach. This can be accomplished by germline gene targeting in mice to inactivate the gene of interest and provides information of the function of the gene in early differentiation. However a lethal phenotype, a developmental block such as observed in *Ikaros*$^{-/-}$ mice or a lack of an organ confining a specialized function such as in *Bcl6*$^{-/-}$ mice prevents more detailed functional analysis of these factors (IV). Therefore, alternative strategies have been developed such as conditional gene deletion in mice, using developmental stage-specific expression of Cre recombinase. Another approach, using the chicken DT40 B cell line has proved to be an important tool to study the function of transcription factors in B cells (IV). DT40 cell line has been instrumental in revealing the molecular mechanisms of BCR signaling. Therefore, analysis of a phenotype where BCR signaling plays a role is justified in DT40 cells. This study expands the current understanding of Ikaros-family transcription factors by showing a novel target gene and a function for Ikaros and Helios in B cells (II). This work reports a counter-regulatory function for Ikaros family members in target gene expression. The mechanism by which this opposite function is achieved at the molecular level remains to be resolved, and warrants further investigation using mutant DT40 cell lines.

This work has provided insight how SHM is regulated and how the temporal regulation of SHM is achieved. The future experiments will reveal the importance of Bcl6-mediated regulation of AID and UNG at the functional level. Importantly, the findings presented here show a previously unappreciated function for Bcl6 as an activator of transcription (II). The genetic complementation after gene inactivation is easily achieved in DT40 cells and thus provides a well-controlled method for loss-of-function studies. The DT40 knockout cell lines also provide platforms to dissect the function of different isoforms of transcription factors and the detailed functional analysis of molecular mechanisms by expressing individual isoforms or mutant forms of the transcription factor on the knockout background (IV). The expression of Pax5 in *Bcl6*$^{-/-}$ cells helped to understand the hierarchy of transcriptional regulatory network (III).

The use of *Bcl6*$^{-/-}$ DT40 cells has identified additional mechanisms how Bcl6-mediated suppression of plasma cell differentiation can be achieved. Taking advantage of the immunoglobulin secreting phenotype of *Bcl6*$^{-/-}$ cells may prove to be useful for *in vitro* production of antibodies. *Bcl6*$^{-/-}$ DT40 cell line provides a novel platform to study the function of plasma cell transcription factors such as IRF4 and Blimp-1 by further gene targeting. Thus, DT40 cell line will continue to be a valuable tool for molecular analysis of network of transcription factors to give results and predictions that will be tested in other model systems.

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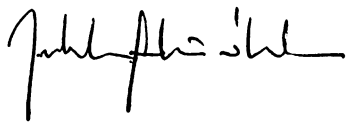

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