Distinct roles for Foxo1 at multiple stages of B cell differentiation

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Distinct roles for Foxo1 at multiple stages of B cell differentiation

A dissertation in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

Sarah Hart Dengler

Committee in charge:
Professor Cornelis Murre, Chair
Professor Karen Arden
Professor Michael David
Professor Robert Rickert
Professor Jean Wang

2008
The Dissertation of Sarah Hart Dengler is approved, and is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2008
Dedication

I would like to dedicate this work to my families the Denglers and the Frickes, especially my parents, BJ and Felton Dengler, and Barbara and Wayne Fricke. They have been an inspiration to me throughout the years.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AID</td>
<td>Activation induced deaminase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>BCAP</td>
<td>B cell adaptor protein</td>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BLNK</td>
<td>B cell linker proein</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB Binding protein</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidly diester</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>CSR</td>
<td>Class switch recombination</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBF</td>
<td>Early B cell factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELP</td>
<td>Early lymphoid progenitor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FDC</td>
<td>Follicular dendritic cell</td>
</tr>
<tr>
<td>Foxo</td>
<td>Forkhead box o</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal center</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>IL-7(R)</td>
<td>Interleukin 7 (Receptor)</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>IRF4/8</td>
<td>Interferon response factor 4/8</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>Jnk</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>LMPP</td>
<td>Lymphoid-primed multipotent progenitor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LT-HSC</td>
<td>Long term hematopoietic stem cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility group</td>
</tr>
<tr>
<td>MPP</td>
<td>Multipotent Progenitor</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>Pax5</td>
<td>Paired box gene 5</td>
</tr>
<tr>
<td>PDK1</td>
<td>3-phosphoinositide-dependent protein kinase 1</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerytherin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll protein</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI(3,4,5)P3</td>
<td>Phosphatidylinositol – 3,4,5- triphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol-3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>SHM</td>
<td>Somatic hypermutation</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>ST-HSC</td>
<td>Short term hematopoietic stem cell</td>
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<tr>
<td>Tc</td>
<td>Cytotoxic T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell Receptor</td>
</tr>
<tr>
<td>TD</td>
<td>T-dependent</td>
</tr>
<tr>
<td>TFH</td>
<td>Follicular B helper T cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TI</td>
<td>T-independent</td>
</tr>
<tr>
<td>T_{reg}</td>
<td>Regulatory T cell</td>
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I would like to thank the past and present Rickert lab with whom it has been a wonderful six years. I would especially like to thank Sidne Omori who was a mentor to me from the beginning and taught me so much throughout the years. I would also like to thank Ravi Kola and Matt Cato who have made the science world light and fun and have put up with all my stressful times in the lab. Ana Miletic Sedy has also been extremely valuable to me in the lab even for the short time I have known her and I appreciate her help very much. And lastly, I would like to give a world of thanks to Gisele Baracho, who entered this lab as the project was near the end, but without whom, I don't think it would ever have been completed. She always gave herself up to her work completely and selflessly and I admire her greatly.

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beer golf. In addition, the Mexico trips, whether for a weekend or a couple of weeks, for surfing or whale watching, with one other person or a group of twenty, were some of the best times of my life. I will cherish those memories forever.

I would like to thank my family. My parents, B.J. and Felton Dengler, have made me believe that I can accomplish anything. Their love and support and because of the amazing people that they are have made me who I am today. My brother and sister-n-law, Sam and Laura Dengler, have always made me strive to want to do better, because they are some of the most amazing people I know. Unlike most people, my family extends beyond blood so I would also like to thank my second family, the Frickes, my California family, the O'neils, my British family, the Margretts, my college family, Catherine and Todd, and my new family, the Rardins for accepting me as part of their families and for all the love and support from them as well. Finally I would like to thank Matt Rardin who has loved and supported me even when I don't deserve somebody as good as him. I am so happy that our lives brought us together, and I cannot wait to enjoy the rest of our years together.

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- **La Jolla Immunology Conference 2007**  
  The Salk Institute, San Diego, CA  
  “A Non-redundant Role for FoxO1 in Early B cell differentiation and Activation”

- **Keystone Symposia Biology of B cells in Health and Disease 2007**  
  Fairmont Banff Springs, Banff, Alberta  
  “A Non-redundant Role for FoxO1 in Early B cell differentiation”

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  Neuroscience Institute, San Diego, CA  
  “A Non-redundant Role for FoxO1 in Early B cell differentiation”

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  Bahia Hotel, San Diego, CA  
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Publications


- Browne C.D., Del Nagro C.J., Cato M.H., Dengler H.S., Rickert R.C. Suppression of PI(3,4,5)P3 production is a key determinant of B cell anergy. Submitted. *Immunity*

- Jaren O.R., Dengler H.S., Chintalapati S., Rickert R.C. PDK1 is required for B cell proliferation, growth and survival. Manuscript in preparation

Work Experience

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ABSTRACT OF THE DISSERTATION

Distinct roles for Foxo1 at multiple stages of B cell differentiation

by

Sarah Hart Dengler

Doctor of Philosophy in Biology

University of California, San Diego, 2008

Professor Cornelis Murre, Chair

The Foxo transcription factors modulate cell fate decisions in diverse systems. Although B-cells express Foxo1, Foxo3 and Foxo4, the importance of individual Foxo members in B-cells is unclear. Here we show that Foxo1 and Foxo3 are not redundant in their roles in B cell development and activation. Foxo1 is shown to play a critical unique role at three stages of B-cell differentiation. Early deletion of Foxo1 causes a severe block at the pro-B cell stage, due to impaired expression of genes involved in B cell development including Pu.1, E2A, and EBF. In addition Foxo1 regulates IL-7R expression blocking cell survival of Foxo1 deficient pro-B cells. Foxo1 inactivation in late pro-B cells results in an arrest at the cycling pre-B cell stage due to a reduction in Rag1/Rag2 expression and subsequent block in recombination of the immunoglobulin
Foxo1 inactivation in transitional B-cells leads to alterations in B1 cell differentiation but normal marginal zone development. In addition, Foxo1 inactivation leads to failed class switch recombination due to impaired Aicda upregulation. Thus, Foxo1 uniquely regulates a transcriptional program that is essential for early B-cell development and peripheral B-cell function.
Chapter 1: Introduction

Overview of the Immune System

The immune system is a highly complex network of cells and immune factors necessary to protect organisms against a vast array of pathogens. It is composed of the innate and adaptive responses that have immediate and sustained responses to pathogens, respectively. The first response to pathogens is the innate immune system that consists of a number of defenses including physical barriers, complement, cytokines, and effector cells such as macrophages, dendritic cells, granulocytes, mast cells, and natural killer cells. The innate effector cells express complement receptors and Fc receptors that detect complement and antibodies attached to pathogens, and through toll like receptors that recognize pathogen associated molecular patterns such as lipopolysaccharides, and methylated DNA. Activation of these receptors induces initial clearance of pathogens through phagocytosis or cell lysis, as well as through activation of the adaptive immune system.

The adaptive immune system responds to specific antigens by both cellular and humoral processes, which act cooperatively to allow additional clearance of pathogens. In addition, the adaptive immune system is unique in that it allows immunological memory in the case of secondary exposure to pathogens. The coordinated activation of the innate and adaptive immune system is necessary to protect an organism, yet a balance of activation and repression of the immune system is also necessary to guard against autoimmunity and immune-associated cancers such as leukemias and lymphomas.
Adaptive Immune System

While almost all organisms possess some form of an innate immune system, the adaptive immune system evolved to allow for pathogen specific immune surveillance and immunological memory. It is composed of a cellular and a humoral branch of defenses in the form of T and B lymphocytes, respectively, which are important for responding to specific pathogens through antigen recognition receptors. During development, lymphocytes undergo a series of DNA rearrangements of antigen receptor genes to produce an almost infinite number of antigen specific receptors. Each B or T cell produces one specific antigen receptor on its surface, creating a population of lymphocytes that can recognize a large array of antigens to efficiently clear pathogens. Upon encounter with a pathogen, lymphocytes undergo clonal expansion of antigen-specific cells that then undergo cellular differentiation into effector cells to allow for clearance of the pathogen. In addition, lymphocytes have the unique function of producing pathogen specific long-lived memory cells, which allows for rapid clearance of a second encounter with the pathogen.

B and T cells vary in their development and function. While B cells develop in the bone marrow to recognize protein, carbohydrate and nucleic acids through their membrane-bound Ig receptor (B cell receptor or BCR), T cells conclude development in the thymus and can only recognize short protein sequences found in the context of a major histocompatibility protein (MHC) found on the surface of an antigen presenting cell (APC). T cells can be divided into a number of subsets, including the two classical cytotoxic T cells (Tc) that provide viral immunity through lysis of infected cells and helper T cells (Th) which upon activation differentiate into Th-1, Th-2, or Th-17 effector cells that function to activate other immune cells such as Tc and B cells through the secretion of cytokines or through cell-cell interactions. Additionally, regulatory T cells
(T-regs) function to dampen T cell activation through the production of anti-inflammatory cytokines, balancing the effector functions of Tc and Th cells to prevent autoimmunity.

B cells function as both APCs to T cells through internalization of antigen through its BCR and as antibody secreting cells through differentiation into plasma cells. Secreted antigen-specific antibody binds to and assists clearance of pathogens through activation of the complement pathway or by allowing recognition and endocytosis of pathogens by cells of the innate immune system.

B cell development

All cells of the immune system originate from self-renewing hematopoietic stem cells found in the fetal liver or the adult bone marrow. HSCs undergo a series of basic lineage decision steps to eventually give rise to a defined immune cell type (Figure 1). In the case of B cells, HSCs initially lose their self-renewal ability and differentiate into long-term and then short-term progenitors (LT-HSCs and ST-HSCs) as the cells upregulate CD34 [1-4]. MPPs further lose their ability to differentiate into erythroid or megakaryocyte cells upon upregulation of the key tyrosine kinase receptor Flt3 and are defined as lymphoid-primed MPPs (LMPPs), which still have the ability to differentiate into myeloid or lymphoid cells [2, 3]. LMPPs next differentiate into early lymphoid progenitors (ELPs) with the upregulation of the recombinase activating genes, Rag1 and Rag2, and into common lymphoid progenitors (CLPs) with the upregulation of the cytokine receptor, IL-7R, as cells become more committed to the B cell fate [5, 6]. Finally, CLPs upregulate B220 and differentiate into pre-pro B cells or Hardy Fraction A (Fr. A) B cells where they begin their development as functional B cells [6]. While each step of the lineage commitment pathway is thought to be composed of a linear path of
commitment to a single cell lineage, the mechanism and plasticity of that commitment are not fully understood. A number of key transcription factors have been found to be necessary for the differentiation of B cells from HSCs and manipulation of theses factors has proved their importance for differentiation and maintenance of cell lineages.

The earliest major transcription factors known to be important for B cell development are PU.1 and Ikaros [4]. Deletion of PU.1, a hematopoietic-restricted ETS transcription factor family member, or Ikaros, a zinc finger domain containing transcription factor, in HSCs leads to a complete block in B cell development prior to the pro-B cell stage [7-11]. The defect in B cell development in the absence of PU.1 or Ikaros is attributed to a decrease of expression of Flt3 as Flt3 is essential for lymphoid development and Flt3L deficient HSCs also show impaired development of B cell precursors [4]. In addition, Flt3, PU.1, and Ikaros deficient animals have a loss of lymphoid and myeloid cells suggesting that they regulate HSC development into MPPs and LMPPs rather than development into B cells specifically [8, 12-14]. Additionally, graded expression of PU.1 directs cells to differentiate into alternate cell fates with lower levels of PU.1 directing cells toward B cell commitment and high levels directing them towards myeloid commitment [15]. Therefore the expression of PU.1 must be tightly regulated to induce B cell lineage development.

After differentiation into CLPs, the key B cell transcription factors, E2A, EBF, and Pax5, are necessary for differentiation into pro-B cells. E2A, a helix loop helix transcription factor, regulates expression of the transcription factors, EBF and Pax5, the key cytokine receptor, IL-7Rα, and many necessary B cell specific genes including cd79a, cd79b, lambda-5, and VpreB [16]. In EBF deficient mice, a similar defect in B cell development and gene expression is seen, and ectopic expression of EBF in E2A deficient CLPs restores expression of all of these B cell specific genes with the
exception of IL-7R expression [16-18]. EBF can also rescue B cell precursor development from Ikaros or PU.1 deficient HSCs, but still does not rescue expression of IL-7Rα [19, 20]. Overexpression of Pax5, a paired domain protein known to be necessary for the expression of the B cell coreceptor, CD19, does not rescue B cell precursors in the absence of E2A, EBF, or PU.1 and therefore does not have the same function in B cell commitment as EBF or E2A [18, 19]. Instead, expression of Pax5 has been shown to be required for repression of alternative lineage fate choices by both promoting B cell lineage genes and inhibiting expression of other lineage specific genes [21-23]. While it has been shown that B cell commitment can be mediated by EBF in the absence of Pax5, it is likely that the 2 transcription factors work coordinately to produce optimal B cell commitment as Pax5 has been shown to bind to the EBF promoter and EBF binds to the Pax5 promoter in a positive feedback loop. Additionally, both act coordinately to induce full expression of mb1 [24-26].

Following B cell commitment, progenitor B cells rearrange the variable regions of their heavy and light chain genes of the immunoglobulin loci to produce a functional B cell receptor. Rearrangement of these genes is regulated by the heterodimer complex Rag1 and Rag2, which function by localizing 2 distal recombination sequences, excising the intervening DNA, and ligating the two regions together with the assistance of DNA double stranded break repair machinery. To allow for allelic exclusion of B-cells, rearrangement of the heavy and light chain must occur in tightly regulated steps beginning with the heavy chain locus and proceeding to the light chain locus. The variable region of the murine heavy chain locus is composed of multiple copies of V, D, and J regions each containing recombination sequences. When low levels of Rag are initially expressed at the LMPP stage, the D and J regions begin rearrangement prior to B cell commitment and can be seen in non-B cell lineages [27,
Later, at the pro B cell stage rearrangement continues from V region to DJ of the heavy chain locus.

Once a functional heavy chain is rearranged, it pairs with the surrogate light chain composed of VpreB and lambda 5 and is expressed as a pre-BCR on the surface of the B cell. The expression of the pre-BCR induces allelic exclusion through the down regulation of Rag and decreased accessibility of the heavy chain locus, and Pre-BCR expression also induces clonal expansion of the large pre B cells [29-32]. At the pre-B cell stage of development, IL-7R is downregulated and expression of IRF4 and IRF8 downregulate the pre-BCR components to allow for further rearrangement of the light chain of the immunoglobulin receptor [31, 33]. Upon IL-7R and pre-BCR downregulation, Rag is re-expressed and light chain rearrangement begins. The light chain is encoded by either of two separate loci, kappa or lambda, each containing only V and J regions that are rearranged beginning with the kappa chain. Upon completion of kappa and possibly lambda rearrangement, a functional IgM molecule is expressed on the surface of the B-cell.

While a functional B cell receptor is the main goal of B cell development, a number of regulatory steps must be taken to ensure that the heavy and light chain are rearranged correctly and that no auto-activation occurs through the receptor binding to a self-ligand. While this is similar to the known positive and negative selection of a functional yet not-autoreactive TCR in T cells, little is known about how BCR selection is regulated in B cells. In B cells, functional rearrangements of heavy and light chain are necessary for B cell development and positive selection. It has been shown that in the absence of Rag, B cells remain at the pro B cell stage although their survival and proliferation at this stage remains unimpaired [29]. If cells fail to express the pre-BCR due to a defect in surrogate light chain expression, B cell development is severely
blocked at the pro to pre B cell stage [34-36]. In addition, light chain deficient animals have a block in the production of immature B cells but have normal numbers of pro and pre B cells [37]. Therefore the inability to produce a functional B-cell receptor blocks B-cell development.

In addition, the random nature of V(D)J gene rearrangement leads to a high incidence of self-reactive B-cells [38]. In regards to negative selection, autoreactive B cells have 3 main strategies for B cell tolerance; secondary rearrangements, apoptosis, and anergy. If a B cell produces an autoreactive BCR, it can downregulate expression of its BCR and undergo further rearrangements of the kappa and lambda light chain that will pair with the heavy chain to produce a non-autoreactive BCR [39, 40]. Additional options for autoreactive B cells are to undergo apoptosis or to downregulate its BCR and escape into the periphery as a tolerant B cell [41]. These processes can be seen in a number of transgenic mouse models including the anti-HEL and the kappa macroself antigen transgenic mouse lines [42, 43]. In the anti-HEL transgenic mice, if the mouse also expresses surface-bound HEL, apoptosis occurs and B cells do not complete development and do not enter into the periphery [43]. If HEL is expressed in a soluble form, B cells down regulate their BCR and enter the periphery as anergic B cells [43]. In the kappa macroself antigen mouse, a synthetic antigen reactive to Ig-kappa is expressed ubiquitously on cell surfaces. Developing B cells expressing a kappa light chain bind the superantigen, inducing downregulation and further rearrangement of the light chain into lambda light chain expressing B cells [42]. Additionally, pre-B cells may also undergo a negative selection checkpoint of autoreactive µ-heavy chain receptors [44]. In surrogate light chain deficient mice some B cells can undergo light chain rearrangement in the absence of first expressing a pre-
BCR. These mice have increased anti-nuclear antibody in the serum due to escape from a pre-BCR checkpoint that would detect the auto-specific \( \mu \)-heavy chain.

The mechanism of B cell positive and negative selection are still under investigation. Basal signaling downstream of the expression of a surface bound pre-BCR or a BCR has been shown to be important for positive selection of B cells producing a functional B cell receptor and downregulation of RAG, but the balance of signaling coming from a functional BCR and an autoreactive BCR is not well understood [45]. Additionally, the defining signals that induce one of the 3 choices of tolerance for an autoreactive B cell (cell death, anergy and secondary rearrangement) is still under much investigation.

B cell activation

B cells can be activated \textit{in vivo} in either a T cell independent (TI) manner or in a T cell dependent (TD) manner. T-independent activation of B cells occurs through a mitogenic signal independent of BCR specificity such as with LPS stimulation (TI-1) or with multiple ligations of the BCR to a polymeric antigen (TI-2). Immunization with a TI-1 antigen does not induce class switch recombination or germinal center (GC) formation. Immunization with a TI-2 antigen induces activation of B1 and marginal zone B cells and can induce germinal center independent class switch to IgG3 isotypes. Activation from either antigen does not induce immunological memory or affinity maturation but induces short-term primary plasmablast formation for the production of IgM (or IgG3 in the case of TI-2 stimulation) low affinity antibody.

As the name implies, T-dependent activation of B cells requires T-cell priming to promote the activation of B cells. Upon activation in a T-dependent manner, B cells can either undergo differentiation into short-term primary plasmablast similar to what
occurs upon T-independent activation, or to enter into a germinal center reaction (Figure 2). The germinal center allows for the interaction of T cells, B cells, and follicular dendritic cells where T cells induce the activation of B cells through cytokine production (IL4 and ICOSL) and CD40 stimulation. This stimulation in the presence of antigen found on the surface of FDC’s induces down regulation of B cell surface IgM and induces class switch recombination (CSR) and somatic hypermutation (SHM) to allow for production of high affinity class-switched antibody. The processes of CSR and SHM are obtained through DNA alterations by the nucleotide deaminase protein AID although the process by which AID is activated and how it induces these changes is still not clear [46-48]. SHM allows for the random mutation of variable regions in the immunoglobulin DNA to allow for the production of high affinity antibody. The selection for B cells expressing high affinity mutations rather than mutations that produce lower affinity antibodies is selected for through the FDC network that presents antigen through Fc and complement receptors [49-51]. Additionally B cells with high affinity antibody elicit help from follicular B-helper T cells (T\textsubscript{FH}) that localize to the site of B cell selection. Presentation of antigen by B-cells induces further T\textsubscript{FH}-dependent activation of the high-affinity B cells [52-54]. Low affinity antibody receptors will undergo apoptosis due to a lack of signal induced downstream of the mutated surface antibody.

Positively selected B-cells exit the germinal center and can either remain as memory B cells for quick protection against re-exposure to the antigen, or they can differentiate into long lived plasma cells that hone to the bone marrow and continually secrete low levels of isotype switched antibody for efficient activation of a memory response.

The regulation of cell fate decisions during T-dependent responses is still being elucidated. Plasma cell versus class switch fate seems to be dependent on the PI3K
pathway and alterations in a number of transcription factors [55]. Over-expression of
Pu.1, which is normally expressed at low levels in mature B cells, blocks class switch
recombination [56]. Similarly, activated B cells overexpressing a major E2A
antagonist, Id3, fail to induce AID expression, suggesting that E2A regulates the Aicda
gene [57, 58]. In addition IRF-4 is known to activate AID and regulate plasma cell
generation [59, 60]. Plasma cell formation also requires the transcription factor Blimp1
and the downregulation of Pax5 [61-63]. How all these signals and transcription
factors interact has still not been fully elucidated due to the early block in B cell
development upon their deletion.

BCR Signaling

Signal induction in B cells initiates through BCR association with antigen,
inducing aggregation of receptors and activation of the Igα/Igβ heterodimer. Igα/Igβ are
constitutively associated with the BCR, and upon aggregation, are phosphorylated on
their immunoreceptor tyrosine-based activation motifs (ITAMs) by the Src kinase family
members Lyn and Syk [64-66]. Phosphorylation of the Igα/Igβ ITAMs induces full
activation of Lyn and Syk, which activate a number of signaling pathways. Syk has
been shown to phosphorylate the B-cell adaptor protein (BCAP), the scaffolding protein
BLNK, and the tyrosine kinase Btk, while Lyn phosphorylates the BCR co-receptor
CD19 [67, 68]. Phosphorylation of BLNK induces association and activation of PLCγ,
inducing calcium flux and activation of PKC and NFκB [66-69]. In addition,
phosphorylated BLNK induces activation of Grb2 and Vav which activate the Erk and
Jnk signaling pathways [70-72]. Btk phosphorylation by Syk initiates its activation,
although full activation is also dependent on localization to the plasma membrane by
binding to phosphatidylinositol-3,4,5 triphosphate (PI(3,4,5)P3) through its PH domain [73-75].

*PI3K signaling in B cells*

Production of PI(3,4,5)P3 is dependent on PI3K, which phosphorylates the phosphatidylinositol-4,5 biphosphate (PI(4,5)P2) at the 3’ position on the inositol ring to produce PI(3,4,5)P3. PI(3,4,5)P3 is embedded in the plasma membrane and can colocalize downstream PH domain containing proteins to the plasma membrane. Class IA PI3K contains both a regulatory (p85) and catalytic domain (p110). In B cells the major regulatory domain, p85α associates with phosphorylated tyrosine residues in YXXM motifs through its SH2 domain. Association of the regulatory domain localizes the catalytic subunit p110δ to the plasma membrane, inducing production of PI(3,4,5)P3. PI3K is activated in 2 separate pathways downstream of BCR signaling (Figure 3). Directly downstream of the BCR, Syk phosphorylation of BCAP and Gab and Cbl on their YXXM motif induces activation of PI3K [76-78]. Additionally, phosphorylation of two key tyrosine residues on the BCR co-receptor CD19 induces PI3K activation [79-83]. Both BCAP deficient and CD19 deficient B cells, as well as B cells expressing a CD19 receptor with mutations of the two tyrosine residues responsible for PI3K activation, have reduced PI(3,4,5)P3 production [83-85]. Additionally, B cells deficient in both CD19 and BCAP expression have almost complete ablation of PI3K activation [86].

Activation of PI3K and production of PI(3,4,5)P3 leads to the activation of a number of signaling pathways through PH-domain containing proteins. As mentioned previously, Btk is dependent on PI(3,4,5)P3 for its full activation [73-75]. In addition PI3K induces activation of PDK1 by binding to PI(3,4,5)P3 through its PH domain.
PDK1 in turn phosphorylates and activates Akt, p70S6K, and PKC isoforms [87-93]. Activation of Akt is also dependent on binding PI(3,4,5)P3 and it induces phosphorylation and inactivation of pro-apoptotic and cell-cycle regulators including Bad, p21, p27 and the Foxo family of transcription factors (Figure 3) [94-101].

The PI3K pathway is important for peripheral B cell differentiation and activation. Deletion of CD19, BCAP, or components of PI3K in B cells leads to a block in B cell differentiation and activation, suggesting, that this pathway is a necessary component in BCR-dependent signaling [84, 102-104]. Deletion of BCAP leads to a decrease in the number of mature B cells and a block in the generation of B1a B cells in the peritoneal cavity. In addition BCAP deficient mice have impaired TI-II responses [85]. Deletion of CD19 in B cells leads to decreased total B cells in the periphery with a specific absence of marginal zone B cells as well as germinal center B cells after T-dependent immunizations [102]. B cells lacking the p85α subunit of PI3K have reduced B cells in the periphery, a slight block at the pro B cell stage, decreased B1a cells in the peritoneal cavity, and are unable to amount a T-independent immune response [84]. In addition mice lacking p110δ also have a slight block in B cell development, decreased B cells in the periphery and decreased marginal zone and B1 B cells, and p110δ deficient B cells also have impaired immune responses to both T-dependent and T-independent antigens [104]. All these mouse models have decreased immunoglobulin in their serum, suggesting that they do not form functional plasma cells upon encounter with antigen. In addition the PI(3,4,5)P3 phosphatase PTEN has also been shown to play a role in B cell development and activation [105, 106]. Deletion of PTEN in B cells induces increased B cell numbers in the periphery as well as increased B1 and marginal zone B cells. PTEN deficient B cells form germinal
centers in response to T-dependent antigen activation and secrete antibody specific IgM upon activation, but do not undergo CSR [105, 106].

In addition to the defect in development seen in the deletion models, PI3K has been shown to be important in a number of different aspects of early B cell development. IL-7R studies have shown that the regulatory subunit of PI3K can bind to phosphorylated tyrosine residues on the cytoplasmic tail of IL-7Rα that are required for signaling downstream to induce proliferation of pro and pre-B cells, but is not involved in inducing further differentiation of the cells [107]. In addition, PI3K is important in tonic signaling from initial expression of functional pre-BCR and BCR. Tonic signaling from a functional receptor expressed on the surface induces activation of PI3K and the subsequent down regulation of Rag proteins to halt further recombination [45, 108].

The fact that PI3K deficient animals have a defect in immunoglobulin secretion and PTEN deficient animals have increased antibody production and a defect in CSR suggests that PI3K plays an essential role in B cell activation and terminal differentiation decisions. In addition, activation of B cells with high affinity antigen induces plasma cell differentiation while stimulation with a low affinity antigen biases towards the germinal center reaction [109], although the signaling mechanism behind this decision is not shown. Our lab has previously shown that the defect of CSR seen in the absence of PTEN can be partially rescued by inhibiting PI3K, consistent with the negative regulatory role of PI3K in CSR [55].

Downstream of PI3K, the BCR signaling pathway forks between Btk dependent signaling and PDK1 dependent signaling events. Deletion of Btk or expression of a mutated Btk lacking its PH domain impairs B cell maturation and activation suggesting a major downstream role for Btk in B cell PI3K signaling [75, 110]. While no work has
been published on the effects of PDK1 deletion on B cell development and activation, initial work in our lab suggests that PDK1 is indispensable for B cell development as well. In addition deletion of \( PDK1 \) in T-cells completely blocks thymic development prior to CD4 or CD8 expression. Downstream of PDK1, activation of Akt, leads to phosphorylation and inhibition of a number of pro-apoptotic and cell-cycle regulators including Bad, p21, p27 and the Foxo family of transcription factors. The effects of loss of Akt has been difficult to study though due to the expression of 3 isoforms in mammals, all expressed in B cells. Therefore, how these PI3K signaling components influence B cell development and activation are still not fully known.

**Foxo factors**

The Foxo (forkhead box o) family of transcription factors is a downstream regulator of the PI3K pathway. Foxos are conserved evolutionarily from the daf-16 isoform found in \( \text{c. elegans} \) through mammals and contain a forkhead winged-helix DNA binding domain. The forkhead domain is conserved throughout the Fox families, which consists of a number of different families that have fairly conserved sequence similarity within the family and are designated by their last letter (foxo versus foxa, foxn, etc). In mammals, Foxos consist of 4 transcription factors, Foxo1, Foxo3, Foxo4, and Foxo6. Foxo1, Foxo3, and Foxo4 are all regulated by the PI3K pathway while Foxo6 is not. The forkhead domain of each are well conserved, and Foxo1, 3, and 4 all contain 3 conserved Akt phosphorylation sites. They also all contain both a nuclear localization sequence and nuclear export signal with one of the Akt phosphorylation sites located within the nuclear export signal (Figure 4a). When Akt is activated by the PI3K pathway, it phosphorylates Foxo factors and induces its binding to 14-3-3 and subsequent transport out of the nucleus to the cytoplasm. In this way Foxo
transcriptional activity is negatively regulated by the PI3K pathway. Foxos act as transcriptional regulators of cell cycle through upregulation of p27 and downregulation of cyclin D2, regulation of apoptosis through upregulation of the pro-apoptotic genes, Bim and FasL, and regulation of reactive oxygen species (ROS) through upregulation of MnSOD and GADD45 (Figure 4b).

In contrast to inactivation of Foxos by PI3K, activation of JNK through cellular stress has been shown to induce the nuclear localization and subsequent transcriptional activity of Foxos. Foxos can also be regulated by a number of other means including ubiquination and acetylation. Poly-ubiquination of Foxos occurs after nuclear export and leads to Foxo degradation [111]. In addition mono-ubiquination of Foxos has recently been found to occur after cell stress and induces the nuclear localization of Foxos [112, 113]. Acetylation of Foxos by p300 and CBP reduces its DNA-binding ability and thus reduces its regulation of target genes [114, 115]. In contrast, deacetylation by SIRT-1 increases its transcriptional activity on stress resistant gene expression while decreasing its transcriptional activity on genes involved in apoptosis [116, 117].

The Foxo core DNA binding sequence has been determined (5’TTGTTTAC3’) and does not vary among the different transcription factors within the family, suggesting that all family members can bind to and regulate the same targets. Although the DNA binding sequence is conserved in all Foxo members, deletion models suggest that each Foxo has unique roles in different cellular systems. Foxo1 deficient mice are embryonic lethal due to improper vascularization, most likely due to impaired vascular endothelial development [118]. Foxo3 deficient mice are viable and have a defect in female fertility due to abnormal ovarian follicular development, and Foxo4 deficient animals have no known phenotypic defect [118].
The variations in the phenotypic defects seen in the absence of various Foxo members can possibly be due to differing expression levels of each Foxo family member in various cell types. In mice and humans, Foxo1 is highly in lymphocytes, Foxo3 is expressed in erythrocytes and ovarian tissues, and Foxo4 is universally expressed (SymAtlas, GNF). In addition to their unique expression patterns Foxos have also been shown to have variation in binding to coactivators that may regulate their targets.

**Foxos in Immunology**

In addition to their roles in other cell systems, Foxos play a role in immune cell development and function. In T cells, deletion of Foxo3 was initially shown to induce a hyper-proliferative response [119]. In addition, deletion of all three Foxos using Mxcre deletion of loxP flanked genes leads to lymphblatic thymic lymphoma [120]. Both cases suggest that Foxos do in fact play a role in cell cycle progression and apoptosis where the loss of one or all three Foxos leads to increase cell survival and aberrant proliferation.

Interestingly, loss of all 3 Foxos also leads to a block in lymphoid cell development in addition to the T-cell defect. Loss of all three Foxos using Mxcre inducible deletion in HSCs leads to a block in generation of CLPs due to impaired protection from reactive oxygen species (ROS) [121]. This suggests that Foxos may play a role in cellular differentiation, which has been studied in other cell types as well including pancreatic β cells, myoblasts, and adipocytes [122-124].

In B cells, initial studies show that Foxos are regulated downstream of BCR signaling by the PI3K pathway. BCR stimulation induces the phosphorylation and nuclear export of Foxos, and it has been shown that BCR stimulation also
downregulates expression of Foxo1 at the mRNA level [125, 126]. Additionally, overexpression of a constitutively nuclear Foxo1 and Foxo3 blocks cell cycle progression and induces apoptosis [125]. Therefore Foxos do play a role downstream of BCR signaling but the unique and redundant roles that Foxo1 and Foxo3 play are still unknown. Recently, overexpression and knockdown studies suggest that Foxo1 and Foxo3 may also play a role in DNA rearrangement of immunoglobulin genes in B cell development [127, 128]. Again this suggests that Foxos also play a role in B cell development, yet the roles that each Foxo may play at this stage are not fully understood.

In this work, we further elucidate the unique and redundant roles of two of these Foxo factors through their deletion in B cells in a mouse model system. We show here that while deletion of Foxo3 has no effect on B cell development or activation, deletion of Foxo1 leads to a number of severe phenotypes suggesting it has unique roles at multiple stages of B cell maturation and activation. Deletion of Foxo1 using three different systems that induce cre expression at a pro-B, pre-B, and transitional stage of development (mb1cre, cd19cre, and cd21cre) induces defects in cell survival, Ig rearrangement, and B cell activation.
Figure 1. Schematic of early B cell lineage development

B cell development outline including known protein markers of lineage classification and necessary transcription factors for each stage of differentiation.
Figure 2. T-dependent B cell activation

Activation of a mature B cell can lead to either primary plasmablast formation and secretion of IgM or to germinal center formation. Proliferation, apoptosis, class switch recombination and somatic hypermutation occur in the germinal center to produce high-affinity class-switched B cells. B cells exiting the germinal center either differentiate into secondary plasmablasts that secrete isotype switched antibody or they persist as memory B cells.
Figure 3. BCR dependent PI3K signaling scheme
Figure 4. Structure and function of Foxo transcription factors

(a) Regulation of Foxo factors through PI3K dependent BCR signaling. In the absence of BCR stimulation, Foxo factors remain in the nucleus, able to regulate transcription. Upon BCR stimulation activated Akt phosphorylates Foxo, inducing its nuclear export, and blocking its transcriptional activity.

(b) Structure of Foxo transcription factors. Each contains a forkhead DNA binding sequence and nuclear localization and export signals. Akt phosphorylation sites are depicted with a red star and CBP acetylation sites are depicted with green stars.
Chapter 2: Foxo1 in early B cell development

Introduction

B cell commitment, survival, and immunoglobulin assembly are all products of a carefully orchestrated network of transcription factors [129, 130]. Development of B cells depends on graded expression of the transcription factors PU.1 and Ikaros in HSCs, which direct development away from the myeloid lineage and into common lymphoid progenitors (CLPs) [7, 11]. Deletion of either \textit{PU.1} or \textit{Ikaros} in mice leads to a block in HSC differentiation into CLPs and a lack of expression of downstream B cell specific transcription factors. The transcription factors, E2A, EBF and Pax5, are each required for the transition from CLPs to the pro-B cell stage [16, 17, 131]. With respect to the transcriptional hierarchy, E2A regulates expression of \textit{EBF} and \textit{Pax5}, as ectopic expression of \textit{EBF} can rescue B cell-specific gene expression in \textit{E2A}\textsuperscript{-/-} cells [18]. While expression of \textit{Pax5} has been shown to be required for repression of alternative lineage fate choices [23], recent evidence indicates that EBF can mediate commitment to the B cell lineage in the absence of Pax5 [132]. B cells reaching the pre-B cell stage require the functions of IRF4 and IRF8 to cease expression of pre-BCR components and to promote \textit{Rag} expression [33, 133, 134]. A better understanding of the early B cell transcriptional network has evolved over recent years, but the inability of known transcription factors to completely rescue the developmental block(s) observed in gene-ablation models suggests that additional factors and feedback mechanisms exist.

In addition to the regulation by the transcription factor network in B cell development and commitment, the receptors Flt-3 and IL-7R regulate the external signals of the bone marrow microenvironment to induce the signaling cascade for B cell development. \textit{Flt3-ligand} deficient mice have reduced myeloid and B lymphoid
progenitors [135]. In addition, IL-7R deficient CLPs have defects in B and T cell development [136, 137]. Flt3 and IL-7Rα must be tightly regulated in their expression levels but the exact signaling mechanisms of how these cytokine receptors are regulated are still unknown. PU.1 and Ikaros deficient HSCs have defects in Flt3 and IL-7R expression [138, 139]. It is thought the Pax5 dependent downregulation of Flt3 at the pro B cell stage is necessary for B cell commitment and overexpression of Flt3 leads to a myeloproliferative disorder [140, 141]. Overexpression of IL-7Rα also leads progenitors away towards a myeloid cell fate suggesting a necessary tight regulation of these cytokine receptors [142]. Flt3 is also expressed at high levels in many acute myeloid leukemia and acute lymphoid leukemia cases suggesting that it has a major role in survival as well as differentiation [140]. Additionally, IL-7R is known to be involved in survival of common lymphoid progenitors and pro B-cells. Signaling through the IL-7Rα induces activation of STAT5, which is involved in the regulation of B cell differentiation genes, and induces activation of PI3K, which controls cell survival and proliferation of pro-B and pre-B cells [107, 143, 144].

After the differentiation of early pro-B cells, the rearrangement of the heavy and light chain loci of the immunoglobulin genes is the primary focus of producing a functional BCR. Initial regulation of the Rag1/2 genes in heavy chain rearrangement is dependent on E2A [145]. At the stage of the pre-B cell checkpoint, IRF4 and IRF8 control the downregulation of the pre-BCR and induce further rearrangement of the light chain [33]. Some evidence suggests that tonic signaling from the expression of the pre-BCR and BCR induces downregulation of the Rag genes to ensure allelic exclusion [30, 32]. This Rag downregulation is PI3K dependent, although the exact mechanism is unknown [45].
One conserved downstream signaling regulator of PI3K is the evolutionarily conserved Foxo family members. PI3K signaling induces nuclear exclusion of Foxo members and downregulation of the pro-apoptotic and cell cycle regulatory genes that Foxo regulates. In addition to their role downstream of PI3K, Foxo family members have been shown to be important for cellular differentiation in multiple cell lineages [120, 122-124]. Three PI3K regulated Foxo family members, Foxo1/3/4 are expressed in B cells, and while all Foxos have highly similar forkhead binding domains and all recognize similar consensus sequences, the redundant versus unique functions of Foxo family members is still unknown. In this work we explore the unique role in Foxo1 compared to Foxo3 as a transcriptional regulator of B cell development.
Results

Foxo1 is required for IL-7R and RAG expression in pro-B cell differentiation

Although signaling via the PI3K pathway is critical for B cell generation and the antibody response, little is known about control of transcription by PI3K signaling effectors. In recent years the Foxo factors, which are negatively regulated by Akt phosphorylation, have emerged as critical downstream regulators controlling diverse physiologic processes including cell cycle progression, metabolism, apoptosis, and oxidative defense among others [146, 147]. The principal Foxo factors expressed in B cells are Foxo1 and Foxo3. To determine their roles in B cell development and function, mice with loxP sites inserted into the Foxo1 gene (Foxo1<sup>L/L</sup>) were crossed with mice that express cre-recombinase (cre) under the control of the mb1 promoter (mb1<sup>cre</sup>) [121, 148]. Mb1<sup>cre</sup> mice express cre at an early B lymphoid progenitor stage and induce deletion of loxP-flanked (floxed) genes at the earliest pro-B cell stage of development [149]. The Foxo1<sup>L/L</sup>mb1<sup>cre</sup> mice were compared to wildtype (Foxo1<sup>+/+</sup>mb1<sup>cre</sup>) littermate controls as well as to Foxo3 null (Foxo3<sup>−/−</sup>) mice [118].

Peripheral lymphoid organs were analyzed by flow cytometry to enumerate B cell subsets. In the spleen, lymph node, and peripheral blood there was over a 10-fold decrease in the percentage of B cells in Foxo1<sup>L/L</sup>mb1<sup>cre</sup> mice compared to both wildtype and Foxo3<sup>−/−</sup> mice (Fig. 5a). In addition, Foxo1-deficient B cells present in the periphery did not express surface IgM or IgD (Fig. 5a). As has been reported [150], Foxo3<sup>−/−</sup> mice had an increase in total cell numbers, but B cell numbers were not significantly increased compared to wildtype littermate controls (Fig. 5b). Due to the sharp decrease in peripheral B cells in Foxo1<sup>L/L</sup>mb1<sup>cre</sup> mice, we examined the B cell compartment of the bone marrow from Foxo1<sup>L/L</sup>mb1<sup>cre</sup> mice to determine the stage of a possible block in B cell development. Indeed, Foxo1<sup>L/L</sup>mb1<sup>cre</sup> mice showed a decreased percentage of total
B cells in the bone marrow as compared to wildtype (Fig. 6a). A close analysis of the bone marrow also revealed a marked decrease in the percentages of B220+IgM+ immature and mature B cells, and B220+CD43+IgM+ pre-B cells compared to wildtype and Foxo3−/− mice (Fig. 6a). In addition, the Foxo1L/L mb1cre mice had a 2-fold increase in the percentage of B220+CD43+ pro-B cells (Fig. 6a). Based on the expression of different cell surface molecules, pro-B cells can be further subdivided into 3 stages of development known as fractions A, B, and C as cells progress towards the pre-B cell stage [151]. Foxo1L/L mb1cre mice had a significant reduction in the percentages of pro B cells that were CD19+BP1− (Fr. B) and CD19+BP1+ (Fr. C) pro-B cells and an increased percentage of CD19+BP1− (Fr. A) pro-B cells (Fig. 6a,b). Together these results suggest that Foxo1L/L mb1cre pro-B cells are blocked at Fr. A of pro-B cell development. In addition Foxo1L/L mb1cre pro B cells have reduced expression of several B-cell restricted gene products and transcription factors required for B cell generation (Fig3).

A pro-B cell block in development can occur due to two primary types of deficiency. One deficiency is marked by failed IL-7R signaling resulting in increased cell death and a failure of pro-B cells to proliferate, resulting in an inability of pro-B cells to develop further down the B cell pathway [107]. The second deficiency, such as that observed in Rag−/− mice, results in failed pre-BCR assembly and signaling [152]. The earliest stages of B cell development are dependent on IL-7 signaling, with a gradual transition to pre-BCR dependent proliferation and differentiation. In Foxo1L/L mb1cre mice pro-B cells failed to expand in IL-7 conditioned cultures, indicating that these cells could present defects in cell proliferation and or survival (data not shown). Because IL-7Rα is necessary for IL-7 signaling at the pro-B cell stage, IL-7Rα expression was analyzed by flow cytometry. While wildtype pro-B cells expressed high levels of IL-7Rα, Foxo1L/L mb1cre mice did not up-regulate IL-7Rα at the pro-B cell stage (Fig. 8a).
To determine if B cells downregulate IL-7R expression through a negative feedback loop initiated through IL-7 signaling similar to T-cells [153], pre B cells were placed in diluting concentrations of IL-7. IL-7Rα expression increased at lower concentrations of IL-7, similar to what is observed in T cells (Fig. 8b). Because IL-7R can signal both through STAT5 and PI3K, we wanted to determine whether the negative feedback loop was dependent on the PI3K pathway. Addition of the PI3K inhibitor LY294002 to pre-B cells stimulated with IL-7 increased the expression of IL-7R on the pre-B cells (Fig. 8b). Because Foxo1 deficient pro-B cells have impaired IL-7R expression, we wanted to determine if the PI3K regulated IL-7R expression was Foxo1 dependent. Wildtype pre-B cultures were infected with wildtype Foxo1 and a constitutively nuclear mutant of Foxo1 and cells were returned to culture in IL-7. Pre-B cells infected with the mutant Foxo1 increased IL-7Rα expression, but cells infected with wildtype Foxo1 showed no change in IL-7Rα expression possibly due to the exclusion of wildtype Foxo1 from the nucleus due to PI3K/Akt signaling downstream of IL-7R (Fig. 8c and data not shown). Therefore IL-7R expression may also be negatively regulated via a feedback mechanism downstream of IL-7 signaling and involving the PI3K pathway and nuclear exclusion of Foxo1.

To test whether the lack of IL-7R in Foxo1 deficient pro-B cells has an effect on pro-B cell survival, Annexin V expression was analyzed on the surface of pro-B cells from Foxo1<sup>L/L</sup> mb1<sup>cre</sup> and wildtype mice. While wildtype pro-B cells had relatively few (15%) Annexin V<sup>+</sup> apoptotic cells, these cells represented the majority (55%) of the pro-B cell compartment in Foxo1<sup>L/L</sup> mb1<sup>cre</sup> mice, indicating that Foxo1-deficient B cells are prone to apoptosis at this stage of development (Fig. 9a).

To gain insight into the mechanisms of Foxo1<sup>L/L</sup> mb1<sup>cre</sup> pro-B cell death, we analyzed the expression of Bim, the pro-apoptotic BH3-only member of the Bcl-2 family,
which can be activated by cytokine deprivation and has been shown to be implicated in a developmental block at the pro-B stage of development [154]. Flow cytometric analysis of freshly isolated pro-B cells from Foxo1\textsuperscript{L/L}mb1\textsuperscript{cre} mice were found to express increased levels of Bim compared to wildtype (Fig. 9b) suggesting that apoptosis of Foxo1 deficient pro B cells can at least be partially mediated by the pro-apoptotic molecule, Bim. In addition, Foxo1 deficient B cells had decreased expression of the anti-apoptotic molecule BcL-x\textsubscript{L} (Fig. 9c). Overexpression of either Bcl-2 or BcL-x\textsubscript{L} has been shown to attenuate apoptosis, and ectopic expression of BcL-x\textsubscript{L} allows for the expansion and survival of pro-B cells [155]. To determine if early B cell development could be rescued by protecting Foxo1-deficient B cells from apoptosis, Foxo1\textsuperscript{L/L} pro-B cells that do not express cre were expanded in IL-7 then transduced with an MSCV-based retrovirus encoding BcL-x\textsubscript{L}. 2 days later, secondary infections were performed with either empty virus (MIT) or cre-expressing virus (MIT-cre) to eliminate Foxo1. While induced deletion of Foxo1 in the absence of exogenous BcL-x\textsubscript{L} resulted in rapid cell death, Foxo1\textsuperscript{L/L} cells expressing both BcL-x\textsubscript{L} and Cre survived in culture but did not proliferate (Fig. 10a,b). In addition, Foxo1\textsuperscript{L/L} cells expressing Cre and BcL-x\textsubscript{L} showed impaired IL-7R expression and Rag expression (Fig. 10c-e).

In addition to the defect in survival, Foxo1\textsuperscript{L/L}mb1\textsuperscript{cre} pro-B cells failed in expressing normal levels of intracellular \(\mu\) heavy chain as compared to wildtype cells (Fig. 11a). Foxo1\textsuperscript{L/L}mb1\textsuperscript{cre} and Rag2\textsuperscript{\textminus/\textminus} mice showed similar by low expression of \(\mu\) heavy chain at the pro B cell stage (Fig. 11a). To determine if Foxo1\textsuperscript{L/L}mb1\textsuperscript{cre} cells can undergo heavy chain Ig rearrangements, genomic DNA from purified pro B cells was analyzed by PCR followed by Southern blot. While D\textsubscript{H} to J\textsubscript{H} rearrangement was normal, Foxo1\textsuperscript{L/L}mb1\textsuperscript{cre} pro B cells showed impaired V\textsubscript{H} to (D)J\textsubscript{H} rearrangement (Fig. 11b). This is consistent with IL-7R signaling defect since IL-7 has been implicated in the
accessibility of the $V_H$ gene locus [156]. It has also been shown that B cells contain $Rag$ enhancer regions ($E_{rag}$) bearing conserved forkhead binding sites and hence Foxo1 could directly regulate $Rag$ expression [157]. Indeed, we found $F_{oxo1}^{+/}\text{mb1}^{cre}$ pro-B cells had decreased transcription of $Rag1$ and $Rag2$ (Fig. 11c). To determine if Foxo1 could also bind to the $E_{rag}$ region, chromatin was isolated from pre-B cell cultures and subjected to ChIP analysis to assess Foxo1 binding to the three conserved $E_{rag}$ regions bearing conserved forkhead-binding sites. Interestingly, Foxo1 bound to the same sites as FoxP1, suggesting that they co-regulate $Rag$ expression in part by binding to the $E_{rag}$ enhancer (Fig. 11d).

*Foxo1 is required for kappa light chain rearrangement in pre-B cells.*

To determine if Foxo1 is required in pre and mature B cells, $F_{oxo1}^{+/}$ mice were crossed with mice expressing $cre$ recombinase under the control of the $Cd19$ promoter ($CD19^{cre}$) [158]. $CD19$ expression is induced by Pax5 and EBF at the pro-B cell stage, conferring significant yet partial $CD19^{cre}$-mediated deletion in pre-B and immature B cell stages, and nearly complete deletion by the mature stage [149, 158]. Bone marrow B cells from $F_{oxo1}^{+/}\text{CD19}^{cre}$ mice were compared to wildtype ($F_{oxo1}^{+/+}\text{CD19}^{cre}$). The $F_{oxo1}^{+/}\text{CD19}^{cre}$ mice had normal pro-B and pre-B cell compartments with a decrease in the percentage of immature and mature B cells relative to wildtype (Fig. 12a,b). In the periphery, $F_{oxo1}^{+/}\text{CD19}^{cre}$ mice showed an increase in the percentage of B cells in the spleen and peripheral blood and a 50% decrease in the percentage of B cells in the lymph node compared to wildtype (Fig. 13a). Surprisingly, the $F_{oxo1}^{+/}\text{CD19}^{cre}$ B cells had a significant percentage of IgM/IgD− B cells that was not seen in the wildtype mice (Fig. 13a,b). These IgM− B cells also expressed low levels of the B cell maturation markers CD62L and CD23, suggesting they are not mature B cells (Fig. 13c). To
determine if the IgM/IgD- B cells present in the Foxo1^{L/L}CD19^{cre} mice are pre-B cells, splenic B cells were stained for surface IgM and IgD and either intracellular \( \mu \)-heavy chain or \( \kappa \)-light chain. While IgM/IgD- wildtype B cells expressed high levels of intracellular \( \kappa \)-light chain and low levels of intracellular \( \mu \)-heavy chain, suggesting that they are class switched B cells, IgM/IgD- Foxo1^{L/L}CD19^{cre} B cells expressed low levels of intracellular \( \kappa \)-light chain and high levels of intracellular \( \mu \)-heavy chain, suggesting that they are small resting pre-B cells (Fig. 14a). Consistent with these data, IgM/IgD- Foxo1^{L/L}CD19^{cre} B cells also showed a decrease in the amount of \( V\kappa-J\kappa \) rearrangement, as well as reduced deletion of the \( V\kappa-J\kappa \) locus via downstream rearrangement with the RS element (Fig. 14b). These results are consistent with decreased Rag expression and heavy chain rearrangement seen at the pro-B cell stage in Foxo1^{L/L}mb1^{cre} mice.
Discussion

We show here that Foxo1 is unique compared to Foxo3 in its regulation of B cell development. Foxo1 deficient pro-B cells have decreased expression of the transcription factors *Pu.1, E2A, EBF,* and *FoxP1* although they have normal expression of *Pax5*. Interestingly deletion of *Foxo1/3/4* in HSCs leads to a block in CLP development [120]. Although this block is attributed to the disregulation of ROS in the absence of the Foxos, skewing the HSCs towards a myeloid developmental pathway, the expression level of *PU.1* in the absence of all the Foxos has not been explored. Regulation of *PU.1* at the pro-B stage of development by Foxo1 may also play a role in the developmental block seen at the HSC stage. Interestingly, the addition of antioxidant N-Acetyl-L-cysteine to *Foxo1/3/4*^L/L^ *Mx^cre^* mice was sufficient in restoring some CLP differentiation in the absence of Foxo factors, but similar initial experiments in *Foxo1* deficient pro-B cells suggests that ROS disregulation is not the only cause of the block in pro-B cell development.

In addition to the alterations in the transcription factor network, *Foxo1* deficient pro B cells have decreased expression of *IL-7Rα*, blocking their survival at the pro-B stage of development through upregulation of the pro-apoptotic molecule Bim and the downregulation of the anti-apoptotic molecule BcL*-x*<sub>L</sub>. Overexpression of BcL*-x*<sub>L</sub> induces cell survival of *Foxo1* deficient pro-B cells but does not rescue *IL-7Rα* expression, *IL-7Rα* dependent proliferation, or *Rag* expression. How Foxo1 regulates *IL-7Rα* expression is still not known, although one possible explanation is through regulation by *Pu.1* and E2A, which regulates *IL-7Rα* as well as other B cell specific genes. We show here that in addition to the decreased expression of *IL-7Rα* in the absence of Foxo1, overexpression of a constitutively active Foxo1 can upregulate *IL-7Rα* expression in wildtype pre-B cultures and that both decreased concentrations of *IL-*
7 stimulation and PI3K inhibition can upregulate expression of IL-7R suggesting a mechanism of negative feedback control of IL-7R signaling dependent on PI3K and Foxo1. Whether this Foxo1 dependent regulation of IL-7Rα expression is unique to early B cell development or is similar in other IL-7Rα expressing cells remains to be explored.

Finally we also show here that in the absence of Foxo1, heavy and light chain gene rearrangement is greatly impaired at the pro-B and pre-B cell stages, respectively. Recently it has been shown that FoxP1 regulates Rag expression through the Rag enhancer region, Erag [157]. Additionally, we confirm an earlier report that Foxo1 can also bind the same enhancer region; although this may not be the only control Foxo1 has on Rag expression since others have shown that Rag expression is still increased in the absence of this enhancer region and in the presence of Foxo1 [127].

It has been suggested that Foxo1 and Foxo3 may play redundant roles in Rag expression and kappa rearrangement [127, 128]. RNAi knockdown of Foxo1 leads to decreased expression of Rag, and overexpression of a constitutively active Foxo3 leads to increased expression of Rag. We show here that while Foxo1 deficient B cells have decreased expression of Rag and Ig gene rearrangement, Foxo3 deficient B cells have no such defect. In addition, deletion of both Foxo1 and Foxo3 does not have a combined effect on Ig expression levels in the bone marrow or the periphery, suggesting that Foxo3 does not compensate for the loss of Foxo1 (Fig. 15). This discrepancy with the overexpression studies may be due to unique post-translational regulation of Foxo factors, or through different co-transcription factors. Because of the similar binding sequence of all the Foxos the overexpression of a constitutively nuclear Foxo may bypass the unique regulation entailed in its proper function so that it could function as many or all Foxos.
Foxo regulation of *Rag* expression may be dependent on PI3K signaling downstream of functional BCR expression. Upon expression of a functional BCR, *Rag* expression is reduced to ensure for allelic exclusion. Others have shown that this downregulation of Rag is regulated by tonic signaling from the BCR through the PI3K pathway. In the absence of p85α, there is a reduced suppression of *Rag* expression inducing further receptor editing [45]. Downstream of PI3K, Foxo proteins are phosphorylated and become transcriptionally inactive due to translocation out of the nucleus. In our mouse model of Foxo1 deficient B cells, *Rag* expression is reduced prior to the complete formation of a functional BCR. This could lead to important functions for Foxo1 in the mechanism of tolerance and B-cell receptor editing.

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Figure 5. Foxo1 uniquely regulates B cell development

(a) FACS profiles of spleen, peripheral blood, and lymph node from Foxo1$^{+/+}\text{mb}1^{\text{cre}}$, Foxo1$^{+/L}\text{mb}1^{\text{cre}}$, and Foxo3$^{-/-}$ mice to determine B cell (CD19$^+$) percentages (top panel) and IgM/IgD expression (bottom panel).

(b) Absolute numbers of splenocytes and splenic B cells.
Figure 6. Foxo1 regulates pro-B cell development

(a) Percentages of bone marrow B cells, and subsets of immature and mature B cells, pro-B cells, pre-B cells, and subcompartments of Fr. A, B, and C pro-B cells. 
(b) Absolute number of bone marrow cells and B lineage cells. FACS plots are representative of 3 mice/group.
Figure 7. Foxo1 regulates network of B cell specific genes

Semi-quantitative PCR of cDNA obtained from 75% pure MACS sorted Foxo1\(^{+/+}\)\_\_mb1\(^{\text{cre}}\) pro B cells (IgM\_IgD\_CD2\_CD25\_B220\(^{+}\)) and 85% pure MACS sorted Foxo1\(^{L/L}\)\_\_mb1\(^{\text{cre}}\) pro B cells (B220\(^{+}\)).
Figure 8. IL7R is negatively regulated by PI3K dependent IL7 signaling

(a) IL-7Rα expression on pro-B cells from Foxo1+/+ mb1cre (red) and Foxo1L/L mb1cre (blue) mice.

(b) Quantification of MFI analysis of IL7Rα on LY treated pre-B cell cultures in various concentrations of IL-7

(c) Quantification of MFI analysis of IL7Rα expression on pro-B and pre-B cells infected with a control virus or a virus expressing constitutively active Foxo1.
Figure 9. Foxo1 regulates pro-B cell survival

(a) Ann V expression on Fr.C pro-B cells from Foxo1+/−mb1cre (red) and Foxo1+/−mb1cre (blue) mice.

(b) Intracellular Bim expression in pro-B (line) and non-B (shaded) cells and MFI analysis of Bim expression from pro-B and non-B cells (5 mice of each group).

(c) Intracellular BcL-\(x_L\) expression of pro-B (line) and non-B (shaded) cells and MFI of BcL-\(x_L\) expression of pro-B and non-B cells (3-4 mice of each group).
Figure 10. BcL-xL rescues survival of Foxo1 deficient B cells but not proliferation or expression of B cell specific genes

(a) Foxo1+/+ \textit{mb1}^{cre}, Foxo1\textit{L/L} \textit{mb1}^{cre} B220⁺ bone marrow cells were grown in culture with IL-7 for 2 days, infected with MSCV-BcL-xL or left uninfected, followed by infection with MIT or MIT-cre. Cultured cells were counted and analyzed by FACS for the Thy1.1 marker of infection on day 2 and day 5.
(b) BcL-xL infected cells superinfected with either MIT or MIT-cre were labeled with CFSE and cultured for 4 days. FACS analysis of proliferation was determined by CFSE division.
(c) FACS analysis of superinfected cell for IL7Ra expression.
(d) PCR from DNA purified from superinfected cells to detect deletion of Foxo1, κ rearrangement, and cμ control.
(e) RT-PCR from RNA purified from superinfected cells to detect expression of Foxo1, \textit{Rag1}, and \textit{Rag2}. 
Figure 11. Foxo1 regulates Rag expression to induce immunoglobulin gene rearrangement

(a) Intracellular µ heavy chain expression in pro-B cells from Foxo1+/+mb1cre (red), Foxo1L/Lmb1cre (blue), and RAG-/ (shaded gray) bone marrow.
(b) Southern blot analysis of distal VH-DJH rearrangements (VHJ558), proximal VH-DJH rearrangements (VH7183), D-J rearrangements, and control (Cθ) in Pro-B cells.
(c) Pro-B cells were analyzed by semi-quantitative RT-PCR for expression of Rag1, Rag2, Foxo1, and β-actin expression.
(d) Foxo1 binding to the Erag enhancer region was analyzed by ChIP on chromatin from pre-B cells from IL7 cultures using antibodies for Foxo1, E47, and an isotype control, followed by PCR of three regions of the Erag enhancer region. All 3 regions contain putative forkhead binding sites while Erag1 and Erag2 regions contain E-box binding sites.
Figure 12. Foxo1 regulates IgM production

(a) Total B cells, immature and mature B cells, pro-B cells, and pre-B cells from bone marrow of Foxo1<sup>+/+</sup>CD19<sup>cre</sup> and Foxo1<sup>L/L</sup>CD19<sup>cre</sup> mice.
(b) Quantification of bone marrow B cell subsets.
Figure 13. Foxo1 regulates mature B cell development

(a) Enumeration of B cells and IgM and IgD expression on B cells from the spleen, peripheral blood, and lymph node.
(b) Quantification of splenic B cell subsets.
(c) Expression of CD23 and CD62L maturation markers on splenic B cells from Foxo1<sup>+/+</sup>CD19<sup>cre</sup> and Foxo1<sup>L/L</sup>CD19<sup>cre</sup> mice.
Figure 14. Foxo1 regulates κ chain gene rearrangement in pre-B cells

(a) Expression of intracellular μ heavy chain and κ light chain in splenic IgM⁺ (red), IgM⁻ (blue) B cells, and non-B cells (shaded gray).

(b) Southern blot analysis of proximal and distal V₃(D₃)J₃ rearrangement, V₅-J₅ rearrangement, V₅ to RS rearrangement, and c₅ control in IgM⁺ and IgM⁻ B cells. FACS plots are representative of 3 mice/group.
Figure 15. Foxo3 does not compensate in immunoglobulin rearrangement upon loss of Foxo1

FACS analysis of IgM and CD43 expression on B220⁺ Bone Marrow B cells, and IgM and IgD expression on splenic B cells of Foxo1⁺/+CD19cre, Foxo3⁻/⁻, Foxo1L/LCD19cre, and Foxo3⁻/⁻Foxo1L/LCD19cre mice.
Chapter 3: Foxo1 in peripheral B cell maturation and the antibody response

Introduction

As previously mentioned, the PI3K pathway regulates B cell differentiation and B-cell activation. PI3K activation downstream of the BCR and cytokine receptors converts PIP(4,5)2 into the second messenger signal PI(3,4,5)P3. PI(3,4,5)P3 binds to the pleckstrin homology domain of effector proteins including PDK1, Akt, and Btk. Binding of PI(3,4,5)P3 induces co-localization of these proteins to the plasma membrane where they can undergo activation. PI3K activity can be regulated by 2 lipid phosphatases, PTEN, which removes the phosphate on the 3’ position of PI(3,4,5)P3, and SHIP, which removes the phosphate on the 5’ position of the PI(3,4,5)P3 molecule to produce phosphatidylinositol biphosphate (3, 4). Signaling through the PI3K pathway can induce cell survival, proliferation, and cellular differentiation in B cells.

Differentiation of B cells into B1 cells is abrogated in the absence of the regulatory p85α subunit of PI3K, and B1 and marginal zone B cell differentiation are impaired in the absence of the catalytic p110δ subunit of PI3K. Additionally, loss of either BCAP or CD19, the two major regulators of BCR dependent PI3K activation in B cells, leads to a loss of B1 cells or B1 and MZ cells respectively. In the absence of the PI3K regulator, PTEN, there is an expansion of the marginal zone and B1 cell populations and the absence of PTEN can recover the block in differentiation seen in the absence of CD19.

In addition to the defects in B cell differentiation, the PI3K pathway plays a role in B cell activation. In the absence of p85α or BCAP, B cells are impaired in their response to TI-2 antigens, and in the absence of p110δ germinal center production and T-dependent activation are also impaired. CD19 deficient B cells also have impaired T-
dependent and germinal center responses and have defects in affinity maturation. In
contrast, PTEN deficient B cells can form germinal centers, have increased basal
secretion of antibody, but cannot undergo class switch formation.

The defects seen in the systems with class switch recombination and affinity
maturation are due to PI3K dependent regulation of expression of the cytidine
deaminase, AID. AID regulates both class switch recombination and somatic
hypermuation of the immunoglobulin genes through direct alterations of the DNA. The
exact mechanism of how AID is regulated is still under investigation, although our lab
has shown that PTEN deficient B cells have decreased AID expression, leading to the
block in class switch formation. In addition, overexpression of a constitutively active
Foxo1 or inhibition of the PI3K pathway with the p110δ inhibitor IC87114 in PTEN
deficient B cells partially rescues AID expression and class switch recombination.

The transcription factor network that is essential for B cell development also
plays a role in class switch recombination, in addition to its roles in early B cell
development. PU.1, which is downregulated in mature B cells, reduces the ability of
peripheral B cell to switch to IgG1 when it is overexpressed [56]. In addition, E2A can
bind the promoter region of the Aicda gene and induce its expression, while
overexpression of a major E2A antagonist, Id3, blocks AID expression [57, 58]. Lastly,
it has also been shown that IRF4 plays a duel role in peripheral B cell differentiation by
activating AID and regulating plasma cell generation [59, 60]. In addition Pax5, which is
necessary for B cell lineage maintenance at early stages, not only represses XBP-1,
which is necessary for plasma cell formation, but also induces Aicda expression [159,
160].

As we have already shown, Foxo1 is both a downstream regulator of PI3K and
also plays a role in the B cell lineage network of transcription factors. Others have
shown that Foxo1 is phosphorylated and downregulated at both the mRNA and protein level upon BCR stimulation [125, 126], suggesting that it plays a role in B cell proliferation and survival upon activation. While the defects in peripheral B cell differentiation have been attributed to the downstream regulator Btk due to the similar defects in B cell differentiation in its absence, the role of the evolutionarily conserved pathway involving, PDK1, Akt, activation and subsequent Foxo inactivation in B cell differentiation has not been explored. Here we induced deletion of Foxo1 in peripheral B cells to explore the role Foxo1 has on B cell differentiation, activation, and class switch recombination.
Results

*Foxo1 modulates peripheral B cell maturation and is required for class switch recombination.*

The pronounced defects in early B cell development presented in Foxo1<sup>L/L</sup> mb1<sup>cre</sup> and Foxo1<sup>L/L</sup> CD19<sup>cre</sup> mice precluded analysis of peripheral B cell maturation and function. Therefore, Foxo1<sup>L/L</sup> mice were crossed to mice expressing cre under control of the CD21 promoter (CD21<sup>cre</sup>). These mice up-regulate cre during the transitional stage of B cell development, after IL-7-dependent differentiation, Ig gene rearrangement and, presumably, Rag-mediated receptor editing has occurred [149]. Flow cytometric analysis revealed Foxo1<sup>L/L</sup> CD21<sup>cre</sup> mice had a modest increase in the percentage of B cells in the spleen and a decrease in the lymph node as compared to wildtype (Fig. 16a). In this regard the percentage of Foxo1<sup>L/L</sup> CD21<sup>cre</sup> B cells representing transitional IgM<sup>+</sup>IgD<sup>-</sup> CD21<sup>-</sup> and mature B cells IgM/IgD<sup>+</sup> B cells was normal compared to wildtype, although the level of expression of IgM and IgD is slightly reduced (Fig. 16a and data not shown). Similar to Foxo1<sup>L/L</sup> CD19<sup>cre</sup> B cells, Foxo1<sup>L/L</sup> CD21<sup>cre</sup> B cells expressed lower levels of L-selectin inhibiting them from entering the lymph node (Fig. 16a). In the peritoneal cavity, there was a reduction in total B1 cells with a bias towards CD5<sup>-</sup> B1b cells (Fig. 16b).

Consistent with the reduced levels of surface Ig expression, Foxo1<sup>L/L</sup> CD21<sup>cre</sup> splenic B cells exhibited reduced survival in response to BCR crosslinking with anti-IgM F(ab')2 as determined by flow cytometric analysis of cell death (Fig. 17a). The survival defect was dose-dependent and activation induced, since sub-mitogenic amounts of anti-IgM did not reveal differences between Foxo1<sup>L/L</sup> CD21<sup>cre</sup> and wildtype B cells (Fig. 17a). These defects were associated with increased Bim expression, as was the case with Foxo1-deficient pro-B cells (Fig. 17b). The observed effects are BCR-specific as
survival of Foxo1-deficient B cells was similar to wildtype when stimulated with LPS and IL4 (Fig. 17b). Notwithstanding the effects of Foxo1 loss on surface BCR expression, Foxo1 does not appear to be required for B cell maintenance, but may contribute to survival post-activation.

In accord with decreased cell survival, \textit{Foxo1}^{L/L}\textit{CD21}^{cre} B cells showed decreased proliferation in response to low concentrations of anti-IgM F(ab')$_2$ and intact anti-IgM stimulation, while proliferation of Foxo1-deficient B cells was similar to wildtype in response to LPS and IL4 (Fig. 18a). \textit{Foxo1}^{L/L}\textit{CD21}^{cre} B cells also show decreased anti-IgM dependent phosphorylation of Akt and ERK (Fig. 18b) and have decreased calcium flux in response to anti-IgM F(ab)$_2$ stimulation (Fig. 18c). Despite the BCR signaling defects observed \textit{in vitro}, immunization of \textit{Foxo1}^{L/L}\textit{CD21}^{cre} mice with the T-independent type 2 (TI-2) antigen, TNP-Ficoll, elicited abundant production of TNP-specific serum IgM antibody compared to wildtype (Fig. 18d). Na{"i}ve \textit{Foxo1}^{L/L}\textit{CD21}^{cre} mice also have slightly increased poly-reactive natural antibody seen at day 0 (Fig. 18d). This observation correlates with the increased percentage of B1b cells in the peritoneal cavity that are capable of responding to TI-2 antigens.

Strikingly, TI-2 immunization of \textit{Foxo1}^{L/L}\textit{CD21}^{cre} mice produced very low levels of IgG3 antibody compared to wildtype (Fig. 18d). To determine if \textit{Foxo1}^{L/L}\textit{CD21}^{cre} B cells can respond normally to T-dependent antigens, mice were immunized with NP-KLH and levels of serum immunoglobulin were analyzed 7 and 14 days later. Serum antibody from \textit{Foxo1}^{L/L}\textit{CD21}^{cre} mice again showed an increase in NP-specific IgM antibody at days 7 and 14 and an absence of detectable IgG antibody at both days (Fig. 19a). To discern whether the absence of IgG antibody is due to decreased germinal center formation, splenic cell suspensions and tissue sections were analyzed for the presence of germinal center B cells. In both analyses, \textit{Foxo1}^{L/L}\textit{CD21}^{cre} mice showed
germinal center responses to NP-KLH comparable to wildtype, suggesting that the lack of IgG antibody production is due to a defect in class switch recombination (CSR) and not due to defects in T-B cell collaboration (Fig. 19b,c).

We have shown previously that in the absence of PTEN, B cells do not undergo CSR in response to antigen or LPS and IL4 stimulation both in vivo and in vitro, and that this CSR defect can be partially restored by the expression of a constitutively active Foxo1 [55]. To determine if Foxo1-deficient B cells can undergo class switch, cells were stimulated in vitro with LPS and IL4 for 3 days and analyzed for proliferation, CSR, and plasma cell formation. While wildtype cells underwent both CSR and plasma cell formation efficiently upon stimulation, there was a striking decrease in CSR and enhanced plasmablast generation in the absence of Foxo1 (Fig. 20a). These cells also did not undergo class switch upon addition of the PI3Kδ inhibitor IC87114, which we have shown increases CSR in wildtype as well as in PTEN-deficient B cells [55], indicating that the lack of class switch seen in Foxo1-deficient B cells is not due to an indirect effect on PI3K signaling (Fig. 20b). To determine if expression of AID, the master regulator of both CSR and somatic hypermutation, was defective in Foxo1L/L CD21cre B cells, RNA from in vitro stimulated cells was analyzed for Aicda expression. Foxo1-deficient B cells did not up-regulate expression of Aicda or generate post-switch IgG1 transcripts in response to LPS and IL4 stimulation, but locus accessibility leading to germline transcript expression was unaffected (Fig 20c). Thus, the Foxo1-associated defect in CSR is linked to Aicda transcription.
Discussion

Activation of mature B cells causes nuclear exclusion of Foxo1 and downregulation of Foxo1 transcription [125, 126, 161]. Here we show that Foxo1 plays a distinct role in peripheral B cell maturation and the antibody response. Deletion of Foxo1 in transitional B cells using CD21^cre led to a reduction in lymph node B cells, consistent with reduced expression of L-selectin. Peritoneal B cells were also reduced and significantly biased towards the CD5^- B-1b subset, while the marginal zone B cell compartment is intact. These collective findings come as a surprise since our previous analyses of B cell-specific PTEN-deficient mice revealed an expansion of the marginal zone and B-1a subsets in the presence of elevated PI(3,4,5)P3 and, presumably, inactive Foxo factors. As B cell specific PTEN-deficient mice induce an overall increase in PI3K activation, the defect seen in the differentiation of PTEN-deficient B cells could possibly be due to Foxo independent signaling pathways of PI3K. Btk-deficient mice have similar defects to the PI3K deficient mice in terms of B cell differentiation. As PTEN-deficient B cells show an opposite phenotype of the PI3K deficient B cells, these defects in B cell differentiation in the absence of PTEN could be due to overactivation of the Btk pathway and not the PDK1/Akt/Foxo1 pathway.

Foxo1-deficient B cells exhibited reduced BCR expression and consequent defects in BCR signaling, proliferation and survival. Similar to pro-B cells, Foxo1-deficient splenic B cells have increased expression of Bim upon stimulation with high concentrations of anti-IgM F(ab')2. Despite reduced BCR responsiveness and an altered B-1 cell compartment, Foxo1-deficient B cells produced a robust T-independent immune response. Because Foxo1-deficient B cells were skewed towards a B-1b versus a B1-a differentiation pathway, this allowed the mice to respond well to TI-2 immunizations, as B1-b cells are the primary responders to TI-2 immunizations.
Foxo1L/L; CD21cre mice underwent normal germinal center formation and produced abundant antigen-specific IgM upon immunization with TD antigens; however, class switched serum antibodies were not generated. This impairment was not due to impaired T cell help, as TI-2 responses showed the same defect. Moreover, in vitro differentiation assays demonstrated normal or elevated plasma cell generation in the absence of accompanied CSR. These findings were consistent with our previous work demonstrating a negative regulatory role for PI3K in CSR [55]. In addition, we also showed that forced expression of nuclear Foxo1 increased the percent of class switched B cells and expression of Aicda in the presence or absence of a PI3K inhibitor [55]. Here, we provide genetic evidence that Foxo1 exerts an essential and nonredundant role in CSR by up-regulating Aicda expression, as Foxo3 deficient mice upregulate Aicda expression to similar levels as wildtype mice upon T-dependent immunization. Hence, PI(3,4,5)P3 appears to be necessary for the activation and cell cycle entry of resting B cells, but must be attenuated to allow proliferation-dependent CSR to proceed. This view is consistent with findings that strong BCR agonists suppress CSR [162]. Thus, while inactivation of Foxo1 does not strongly impact late B cell maturation and antigen-driven clonal expansion, it serves an essential role in CSR.

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Figure 16. Foxo1 regulates peripheral B cell differentiation

(a) Quantization of total B cells, IgM/IgD-expressing subsets, and CD9⁺CD21⁺ marginal zone B cells in spleens and lymph nodes from Foxo1⁺/⁺CD21⁺cre and Foxo1⁺/⁻CD21⁺cre mice.

(b) Enumeration of IgM⁺CD23⁻ B-1 cells and CD5⁺ B-1a vs. CD5⁻ B-1b subsets from the peritoneal cavity of Foxo1⁺/⁺CD21⁺cre (red) and Foxo1⁺/⁻CD21⁺cre (blue) mice.
Figure 17. Foxo1 regulates cell survival in response to BCR stimulation

(a) Stimulation of purified splenic B cells from Foxo1<sup>T</sup>/CD21<sup>cre</sup> (red) and Foxo1<sup>L/L</sup>CD21<sup>cre</sup> (blue) mice with titrated amounts of anti-IgM F(ab')<sub>2</sub> (48 hours) and measurement of cell viability using 7AAD.

(b) Western blot analysis for Bim expression in purified splenic B cells stimulated overnight with or without 10 µg/mL of anti-IgM F(ab')<sub>2</sub>. FACS plots are representative of 3 mice/group.
Figure 18. Foxo1 regulates anti-BCR response \textit{in vitro} but not antibody response \textit{in vivo}

(a) Proliferation measurements of purified splenic B cells from Foxo1\textsuperscript{+/+}CD21\textsuperscript{cre} (red) and Foxo1\textsuperscript{L/L}CD21\textsuperscript{cre} (blue) mice following CFSE labeling and stimulation (3 days) with 1\,$\mu$g/mL or 10\,$\mu$g/mL of either anti-IgM F(ab\textquotesingle)\textsubscript{2} or intact anti-IgM in the presence or absence of IL4; or stimulated with LPS and IL4. CFSE partitioning was analyzed by FACS.

(b) Western blot analysis of phospho-Akt and phospho-Erk from anti-IgM F(ab\textquotesingle)\textsubscript{2} stimulated splenic B cells.

(c) Calcium flux by purified splenic B cells from Foxo1\textsuperscript{+/+}CD21\textsuperscript{cre} (red) and Foxo1\textsuperscript{L/L}CD21\textsuperscript{cre} (blue) mice loaded with Fluo-4 and stimulated with 10\,$\mu$g/mL anti-IgM F(ab\textquotesingle)\textsubscript{2}.

(d) ELISA to detect TNP-specific IgM and IgG3 present in the serum of Foxo1\textsuperscript{+/+}CD21\textsuperscript{cre} (open circles) and Foxo1\textsuperscript{L/L}CD21\textsuperscript{cre} (filled circles) mice before and 5 days after IP immunization with 10\,$\mu$g TNP-Ficoll in PBS.
Figure 19. Foxo1 is required for class switch recombination but not germinal center formation

(a) ELISA to detect NP-specific IgM and IgG present in the serum of Foxo1\textsuperscript{+/+}CD21\textsuperscript{cre} (open circles) and Foxo1\textsuperscript{L/L}CD21\textsuperscript{cre} (filled circles) mice before and 7 and 14 days after IP immunization with 100 µg of NP-KLH in alum.

(b) Splenic GL7\textsuperscript{+}Fas\textsuperscript{+} germinal center B cells were quantified by FACS, and

(c) Visualized by fluorescent imaging of frozen sections by PNA (green) and B220 (red) (D14 only). FACS plots are representative of 3 mice/group.
Figure 20. Foxo1 regulates AID expression

(a) Flow cytometric analysis of LPS/IL-4 stimulated (3 days) B cells from Foxo1^{+/+}CD21^cre and Foxo1^{L/L}CD21^cre mice. Proliferation was measured by CFSE partitioning on plasmablasts (Syn1^+) undergoing CSR (IgG^+).

(b) Percentage of class switched cells generated in the absence or presence of the PI3Kδ inhibitor IC87114 (+IC8).

(c) Semi-quantitative PCR to measure AID expression and the generation of γ1 germline transcripts (GLT), γ1 post-switch transcripts (PST), and γ1 circular transcripts (CT-lower band) of in vitro stimulated B cells.

(d) QT-PCR of AID expression of splenic germinal center B cells from Foxo1^{+/+}CD21^cre, Foxo3a^{−/−}, and Foxo1^{L/L}CD21^cre mice 10 days after immunization with 200 µg of NP-KLH in alum.
Chapter 4: Discussion

We show here that Foxo1 compared to Foxo3 is unique in regulation of B cell development and activation. In the absence of Foxo1 pro-B cells have impaired survival, proliferation, and expression of the majority of the members of the B cell transcription factor network. In addition Foxo1 regulates Rag expression such that in the absence of Foxo1, pro-B and pre-B cells do not upregulate Rag and therefore have impaired rearrangement of the heavy and light chains of their immunoglobulin loci. By comparison, Foxo3 deficient B cells exhibited no impaired development. Because both isoforms are expressed in developing B cells, Foxo1 and Foxo3 may play different roles in B cell development. While Foxo1 and Foxo3 have similar forkhead binding domains, nuclear localization and export domains, and Akt phosphorylation sites, little is known about other sites of phosphorylation and acetylation of Foxo1 and Foxo3 that may uniquely regulate their transcriptional activity. In addition, while many studies have determined Foxo-specific targets through overexpression studies, physiologic target genes that are regulated by Foxo may be preferentially regulated by one Foxo protein versus another, which would be distorted in the continuous overexpression of one Foxo factor. As has been seen in Rag regulation, overexpression of a constitutively nuclear Foxo3 results in Rag upregulation, yet the Foxo3 deficient B cells do not have the comparable defect. In addition to preferential binding, Foxo factors may also be regulated by co-transcription factors. These other methods of Foxo regulation remain to be characterized. Here we show clear evidence that Foxo factors do not play redundant roles in development, and therefore more studies are necessary in understanding their unique post-translational regulation and genetic targets.
The role of Foxo1 in B cell development differs from its known function as a cell cycle regulator and inducer of apoptosis. While Foxo factors have been implicated in the differentiation of HSCs, their primary function was thought to be due to regulation of reactive oxygen species, a known common role for all Foxo factors [121]. Here we show that Foxo1-deficient pro-B cells have defects in many transcription factors involved in B cell differentiation. Whether this is a direct regulation of these factors or an indirect mechanism is still under investigation. Interestingly, introduction of the antioxidant N-Acetyl-L-cysteine to Foxo1\textsuperscript{F/F} mb1\textsuperscript{cre} B cells did not recover B cell development in initial experiments, suggesting that the abrogation in the B cell targets may be due to direct Foxo1 regulation, rather than through regulation of ROS [121]. It would be interesting to determine both the effects of loss of only Foxo1 in CLP development from HSCs, and the other B cell specific targets that are abrogated upon loss of all Foxos.

In addition to defects in the expression of Pu.1, E2A and EBF, deletion of Foxo1 results in a lack of Rag expression. Other groups have shown that PI3K signaling downstream of a newly formed BCR induces Rag downregulation and positive selection [45], and here we show evidence that PI3K may signal through Foxo1 in order to turn off Rag expression. This leads to a possible interesting role in B cell positive selection and receptor editing. Future experiments will determine the role of Foxo1 in receptor editing by deleting Foxo1 in systems that induce receptor editing such as the kappa macroself antigen transgenic mouse line [42]. In this system, B cells undergo receptor editing to a lambda light chain to avoid auto-specific recognition of the kappa antigen. In the absence of Foxo1, receptor editing should be impaired due to the loss of Rag expression, and therefore may lead to a better understanding of how B cells undergo different mechanisms of tolerance.
In addition to the roles that Foxo1 plays in B cell development, we show that PI3K signaling downstream of BCR activation blocks class switch recombination through Foxo1 dependent mechanisms. While the model of PI3K activation leading to a block in class switch recombination has been proposed through the PTEN deficient B cells, here we provide direct evidence that Foxo1 regulates AID expression and class switch recombination both in vivo and in vitro. It is unknown whether Foxo1 regulates AID expression directly as the AID promoter and enhancer sequences are still not well defined, nevertheless, there are forkhead consensus sites in the 1kb upstream region of the AID gene. In addition, Foxo1 may regulate AID expression through the same B cell transcription factor network that it regulates during B cell development. It has been shown that abrogation of PU.1 and E2A can lead to blocks in class switch recombination; therefore, it would be interesting to determine if deletion of Foxo1 leads to a disregulation of these transcription factors upon B cell activation, leading to the block in AID expression and class switch recombination.

Interestingly, Foxo1 regulates the two stages of B cell function at which DNA rearrangement occurs. In order for heavy and light chain rearrangement at the early stages of B cell development, and in order to undergo class switch recombination, the immunoglobulin locus undergoes DNA double stranded break and repair. Foxo factors have been implicated in DNA damage repair, induction of apoptosis, and regulation of cell cycle upon DNA damage through the regulation of Cdk1 and Cdk2 [163, 164]. The fact that Foxo1 deficient B cells do not undergo these processes necessitating DNA damage repair, introduces a broader question of its function during these stages. As a cell cycle regulator, Foxo1 may induce an initial block in the cell cycle during DNA damage so that the necessary molecules may repair the DNA. In addition, cell stress involves induction of Foxo dependent gene expression. Therefore, the blocks observed
in the absence of Foxo1 may involve the inability of the cells to cope with DNA damage
and cell stress involved in the process of DNA rearrangement for immunoglobulin
production and class switch recombination. In conclusion, here we show that while all
Foxos are expressed, Foxo1 had unique roles in B cell development, immunoglobulin
gene rearrangement, and class switch recombination. In addition to its role in BCR
signaling, we present evidence here that Foxo1 also regulates the B cell developmental
transcription network, *Rag*, and *aicda* expression.
Methods

Mice

Mice with loxP sites flanking exon 2 of *Foxo1* [120] (*Foxo1*\(^{f/c}\)) were crossed to *CD19-cre* [158] (*CD19\(^{cre}\)\), *mb1-cre* [148] (*mb1\(^{cre}\)\), or *CD21\(^{cre}\) [165] (*CD21\(^{cre}\)\) to generate mice with conditional deletion of Foxo1 at various stages of B cell development. Mice with a gene trap cassette inserted into the first intron of the *Foxo3* gene were used for complete deletion of *Foxo3* (*Foxo3\(^{-/-}\)\) [118]. Mice were bred and housed at the Burnham Institute for Medical Research animal facility and all experiments received IACUC approval. Mice were used at 8-10 weeks of age for peripheral B cell analysis and 4-6 weeks of age for bone marrow cultures.

Flow Cytometry

1.0 million cells in single cell suspensions were first stained with biotinylated and/or FcBlock antibodies in FACS buffer (1% FBS, 0.1% Azide in PBS) for 20' at 4°C, followed by incubation with a cocktail of FITC, PE, PerCPCy5.5, PeCy7, APC, and APC-Cy7 conjugated antibodies. For intracellular staining, the cell surface was stained, eBioscience fixation/permeabilization buffer was used for permeabilization, and intracellular stains were performed. Antibodies used were: biotinylated- BP1, CD9, CD23, CD86, IgD, IgG1, IgG2a/b, IgG3; FITC-AnnV, CD2, CD5, CD21, CD69, GL7, H2Db, IgD, μ, κ, λ, and anti-rabbit; PE-CD138, CD23, IgD, CD43, CD3, and CD127; PerCPCy5.5-B220 and streptavidin; PeCy7- CD11b, CD3, and streptavidin; APC-CD127, B220, CD19, IgM, CD62L, and streptavidin; APCCy7- CD19 and B220 (BD-Pharmingen or eBioscience). Rabbit anti-mouse Bim was purchased from Cell Signaling.
Technology. Data was collected on a FacsCanto (BD) and analyzed using FlowJo software (TreeStar).

**Cell Culture**

Red blood cells from splenic single cell suspensions were lysed using ACK (150 mM NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA) for 5' at 4°C. B cells were purified by negative selection using CD43-labeled magnetic beads (Miltenyi). For CFSE cultures, B cells were labeled with 2.5 µM CFSE for 10 minutes at room temperature in PBS, washed and cultured in C/10 RPMI at 2 million cells/mL in a 96 well flat bottom plate for 3 days with goat anti-mouse-IgM F(ab’)2 (Jackson) or rabbit anti-mouse intact-IgM (Jackson) at 10 µg/mL, 1 µg/mL or 0.1 µg/mL with or without 10 ng/mL IL4, (RnD Systems) or in 20 µg/mL LPS (Sigma) with 10 ng/mL IL4.

**Bone Marrow Cultures**

B cells were positively selected from bone marrow with B220-labeled magnetic beads (Miltenyi). Cells were cultured in OMEM with 15% FBS (Hyclone), 100 U/ml Pen/Strep, 2 mM L-glut, and 55 µM BME. For dilution assays, cells were cultured with the addition of rIL-7 (RnD Systems) at a concentration of 10 ng/mL, 1 ng/mL, 0.1 ng/mL, or 1 ng/mL and with LY-290042 at a concentration of 2.5µM, 1.25µM, 0.625µM, or 0µM. Cells were cultured for 2 days before FACS analysis for expression of IL-7Rα.

**Calcium Flux**

Purified splenic B cells were labeled with Fluo-4 (Invitrogen) for 60’ at 37°C. Cells were washed and resuspended at 2 million cells/mL and collected on the
cytometer for 15 seconds prior to stimulation. Cells were stimulated with 10 µg/mL anti-IgM F(ab’)2 and collected on the cytometer for 3’.

Bone Marrow Infection

Viral supernatants were generated from transfected Phoenix-eco cells as previously described [55]. B cells were positively selected from bone marrow using B220-labeled magnetic beads (Miltenyi). Cells were cultured for 48 hours in OMEM with 15% FBS (Hyclone), 100 U/ml Pen/Strep, 2 mM L-glut, and 55 µM BME with the addition of 5 ng/mL IL-7 (RnD Systems). Cells were resuspended at 1 million cells/mL in <1:2> diluted MSCV-BcL-xL viral supernatants that was previously incubated with 8 µg/mL polybrene. Cells were spun at 2500 rpm for 90’ at 30°C and then placed at 37°C for 60’. Cells were then collected and resuspended in C/15 OMEM with 5 ng/mL IL-7 and cultured for an additional 48 hours, then superinfected with either empty virus (MSCV-IRES-Thy1.1) or MIT-cre. Cells were returned to culture with IL-7 and analyzed 2 and 5 days later

Semi-Quantitative RT-PCR and QT-PCR

Bone marrow pro-B cells were purified by initial depletion of CD25, CD2, IgM, CD11b+ cells by using biotinylated antibodies followed by depletion using anti-biotin beads (Miltenyi). The remaining cells were further purified for B cells using B220+ positive selection (Miltenyi). Resultant B cells were analyzed by flow cytometry for purity (85-90% pro-B). RNA was purified from cells using TRIZOL LS (Invitrogen) according to manufacturer’s protocol. RT reactions were performed using Superscript II first strand synthesis kit (Invitrogen). PCRs were performed on serially diluted cDNA for 30 cycles with primers published in [20]. Aicda QT-PCR was performed on RNA from
purified B cells from immunized mice and was performed as in [55]. Aicda expression was normalized to GAPDH first and then to the percent of Fas\(^+\)GL7\(^+\) germinal center B cells, determined by FACS.

Southern Blot

DNA was purified from FACS-sorted pro-B cells or magnetically labeled splenic B cells using salt extraction method and resuspended at 25 ng/mL in TE buffer. PCRs for \(D_H\) to \(J_H\), distal \(V_H\) (VHJ558) to (D)\(J_H\) (JH4), proximal \(V_H\) (VH7183) to (D)\(J_H\) (JH4), \(V_K\) (V\(K\)Deg) to J\(K\) (MAR35K), \(V_K\) to Recombination Sequence (RS), or constant C\(\mu\) were performed on 100, 25, 6.25, and 1.56 ng/mL for 30 cycles as previously described [59]. Radio-labeled probes were made using Klenow of PCR products generated from wildtype splenic B cell genomic DNA. Southern blotting of heavy and light chain rearrangement were performed as described [166, 167].

Chromatin-IPs

ChIP assay was performed essentially as described for the anti-acetylated histone H3 Ab-based ChIP assay kit (Upstate Biotechnology) with minor modifications. In brief, 37% formaldehyde solution was added directly to pro/pre-B cell cultures to a final concentration of 1% and cells were incubated for 10’ at 37°C. Cells were harvested and washed twice with ice-cold PBS containing protease inhibitors (Roche) and lysed with SDS lysis buffer for 10’ on ice. The lysate was sonicated to an average length of 200-1000 bp and clarified by centrifugation. The samples were diluted 10-fold with ChIP dilution buffer then pre-cleared with protein A/G beads (Calbiochem) for 1 h followed by incubation with either normal rabbit IgG (Jackson), anti-Foxo1 (Santa Cruz or Cell Signaling Technology), or anti-E47 (Becton-Dickinson) overnight at 4°C. Protein
A/G beads were added in the final hour of incubation prior to harvest. After washing and elution of beads per the manufacturer’s instructions, cross-links were reversed by heating at 65°C overnight followed by proteinase K treatment. DNA was purified using the Qiagen PCR purification kit, and PCR reactions were performed on eluted DNA for 30 cycles (94°C, 15”, 60°C, 15”, 72°C, 1’) using previously published primers [157].

Biochemistry

For short-term stimulations, purified B cells were stimulated with 10 µg/mL of anti-IgM F(ab’)2 for the indicated times in PBS at 20 million cells/mL. For overnight stimulations, cells were cultured in C/10 RPMI at 20 million cells/mL. Reactions were stopped by the addition of cold PBS and cells were immediately pelleted and lysed with RIPA (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate and 0.1% SDS) including DNAse, protease, and phosphatase inhibitors. Westerns were probed with anti-Bim, anti-pAkt(S473), and p-ERK (Cell Signaling Technology), and primary antibodies detected with Odyssey system (Licor) using anti-rabbit DyeLight800 (Pierce) or anti-rabbit-680 (Invitrogen).

Immunizations and ELISAs

For T-dependent immunizations, animals were immunized ip with 100 µg of NPKLH precipitated in alum (Imject, Pierce) and serum was collected on day 0, 7, and 14. For T-independent type 2 immunizations, mice were immunized with 10 µg of TNP-Ficoll and serum was collected on day 0 and 5. ELISAs for antigen-specific antibody was performed as described previously [55]. For AID expression, animals were immunized ip with 200µg of NPKLH in alum and analyzed 10 days later.
Immunohistochemistry

Spleens were frozen in OCT (Tissue-Tek) and 8 micron sections were stained with anti-PNA-FITC, and anti-B220-APC (pseudo-colored red with Slidebook).

Statistics

Groups of 3-8 mice were used in statistical analysis. p values were calculated using the student's t-test.
References


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