Defining the Requirement for ShcA in Murine B Cell Development

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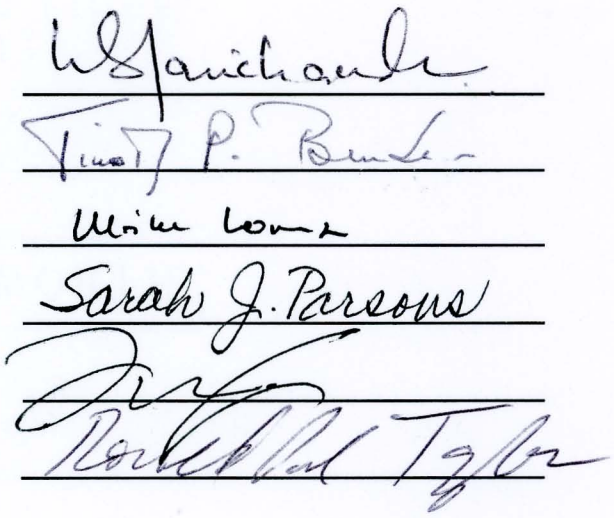
A Dissertation presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

Department of Microbiology

University of Virginia
December 2009



Abstract

The adaptor protein Shc is phosphorylated downstream of many cell surface receptors, including antigen and cytokine receptors. However, the role of Shc in B cell development has not been addressed. Here, through conditional expression of a dominant negative Shc mutant and conditional loss of Shc protein expression, I tested a role for Shc during early B lymphopoiesis. In chapter III, I identified a requirement for Shc beginning at either the transition from the pre-pro-B to pro-B stage or within the pro-B pool, with a strong reduction in the number of pre-B cells. This developmental defect appeared to be due to increased cell death rather than impaired proliferation. Additional studies in chapter IV suggest a role for Shc in IL-7-dependent signaling in pro-B cells. Shc is phosphorylated in response to IL-7 stimulation in pro-B cells, and pro-B cells from mice with impaired Shc signaling display signs of increased apoptosis. Together, these data suggest a critical role for Shc in early B lymphopoiesis with a requirement in early B cell survival. In addition, I also identify Shc as a required player in signaling downstream of the IL-7 receptor in early B cells.

Acknowledgments

First, I would like to thank my advisor, Kodi Ravichandran, for allowing me to join his laboratory. I have thoroughly enjoyed pursuing the project that has culminated in this dissertation. In particular, his "think big" attitude has encouraged me to truly examine my capabilities and reach beyond what I consider to be my limits. Ravi has encouraged me to work independently, and I now feel fully competent to set out on my new career path.

I would also like to thank my co-advisor, Tim Bender. Tim not only introduced me to the Cre mice that revealed a role for Shc during early B cell development, but he has provided essential expertise in the B cell development aspect of my project. Tim's kind words toward my data made me proud of my work, which has been extremely helpful during intimidating times, such as my first AAI conference poster presentation.

I would also like to express my sincere gratitude to my Committee members, Tim Bender, Sally Parsons, Ron Taylor, Ulrike Lorenz, and Loren Erickson. I have been fortunate to have not only an incredibly intelligent Committee but also one that is kind and constructive. My Committee meetings were always positive experiences in which I felt at ease to laugh (often at myself) while my data were thoroughly analyzed and abundant constructive criticisms given. I will always be grateful for the support and time that my Committee members spent in analyzing my project data. These meetings have been extremely influential on my development as a scientist.

During my time in the Ravichandran lab, I have had the privilege of working with a group of highly intelligent and fun individuals. In particular, Rusty Elliott provided guidance during my rotation in the lab, and I have greatly appreciated his help on

multiple levels throughout the years. In addition, Jason Kinchen, Scott Walk, Lisa Haney, Daeho Park, and Amelia Hufford have given insightful scientific advice and friendship, lodging, and dog walking when these were desperately needed! I have also made several strong friendships with people in other labs, and I will miss everyone dearly when I leave.

I would like to thank Colin Brinkman and Joanne Lannigan for sharing their flow cytometry knowledge, which is a core component of this dissertation. In addition, I will always appreciate Colin's support and friendship throughout my years at UVA.

Throughout my life, my parents have constantly encouraged me to achieve my highest goals. They have given me constant love and support. In particular, I feel my mother taught me the ability to persevere. She has encouraged me to become an independent woman, which has also driven me from a grade-school age to be successful. I truly feel blessed to have grown up with so much love and affection from my family.

In addition to my parents, I have gained a wonderful family from my husband, Will Giles. I have felt included in this family since we first began dating so many years ago. Colleen, Danny, and Fred are some of the most generous and loving individuals I have encountered, and I sincerely appreciate their love and support.

Finally, I want to express my dearest and deepest appreciation for my husband, Will. Will's love and support are my foundation and are a constant in my life around which all else rotates. I feel most of my strength is due to my knowledge that I have such and incredible person constantly supporting me. I admire and respect Will more than anyone I have met, and I find myself becoming a better individual simply by trying to keep up with him!

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List of Abbreviations

AD	Activation Domain
BCR	B Cell Receptor
BME	β-mercaptoethanol
bp	Base Pairs
BrdU	5-Bromo-2-Deoxyuridine
BSA	Bovine Serum Albumin
cDNA	Complimentary DNA
CFSE	Carboxy-Fluorescein Diacetate Succinimidyl Ester
CH	Collagen Homology
CLL	Chronic Lymphocytic Leukmia
CLP	Common Lymphoid Progenitor
DNA	Deoxyribonucleic Acid
dCTP	Deoxycytidine Triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DN	Double Negative
DP	Double Positive
ES	Embryonic Stem
FACS	Fluorescence-Activated Cell Sorter
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
FO	Follicular (B cell)

GAP	GTPase-Acitivating Protein
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescence Protein
gMFI	Geometric Mean Fluorescence Intensity
Grb2	Growth Factor Receptor-Bound Protein-2
GST	Glutathione-S-Transferase
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphate Hydrolyase
HC	Heavy Chain
HSC	Hematopoietic Stem Cell
Ig	Immunoglobulin
IL	Interleukin
IP	Immunoprecipitation
IP injection	Intraperitoneal injection
kDA	Kilodalton
Lin	Lineage
LPS	Lipopolysaccharide
LSK	Lin negative, Sca-1 positive, c-kit high FACS-staining cell population
MAPK	Mitogen Activated Protein Kinase
MFI	Mean Fluorescence Intensity
MPP	Multipotent Progenitors
mRNA	Messenger RNA

MZ	Marginal Zone
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PI	Propidium Iodide
PI-3K	Phosphatidylinositol 3-kinase
PIP	PCNA-Interacting Protein
PTB	Phosphotyrosine Binding Domain
RNA	Ribonucleic Acid
RT	Room Temperature
RT-PCR	Reverse Transcriptase PCR
Sca-1	Stem Cell Antigen 1
SH2	Src Homology Region 2
SH3	Src Homology Region 3
SLC	Surrogate Light Chain
SP	Single Positive
T1, T2, T3	Transitional B cell (Number refers to developmental stage)
TCR	T Cell Receptor
XLA	X-linked Agammaglobulinemia

Chapter I

Introduction

B cells are lymphocytes that play a role as effector cells during the humoral immune response. B cell development is a highly ordered process that requires cells to progress through multiple checkpoints during maturation. These checkpoints often require cells to express and transmit signals from cell surface receptors to regulate subsequent decisions regarding survival, proliferation, and differentiation. These checkpoints ensure that nonfunctional as well as potentially autoreactive B cells are removed from the system. Dysregulation of B cell development can lead to lymphopoenic disorders such as X-linked agammaglobulinema (XLA) or lymphoproliferative diseases such as chronic lymphocytic leukemia (CLL), lymphoma, or autoimmune disorders (reviewed in [1-3]). Therefore, it appears essential that B cells are subjected to both positive and negative checkpoints during maturation.

The road to becoming a mature B cell has many requirements, and only a fraction of B cells make it to maturity. Identification of cell surface determinants and advances in flow cytometry have expanded our knowledge of the multiple stages of B cell development from the hematopoietic stem cell (HSC) to the mature B cell (Figure 1-1). These advances have also allowed us to identify specific stages at which proteins are required for B cell development and further our understanding of B lymphopoiesis.

B cell development in the adult mouse

Normal developmental progression in the bone marrow

The HSC to CLP stage

During embryogenesis, B cells are generated in the fetal liver [4]. After birth, the bone marrow takes over as the principle site of B lymphopoiesis [5]. As with all lymphocytes, B cells originate from HSCs. The HSC compartment is made of a heterogeneous population of cells that possess the ability to self renew and can also repopulate all hematopoietic cells when transplanted into a lethally irradiated host. These cells are defined as being negative for cell surface markers expressed on mature myeloid and lymphoid lineages, highly expressing c-kit, and positive for the stem cell antigen Sca-1.

Several intermediates have been identified between the HSC and common lymphoid progenitor (CLP) cell stage. Stem cells and early progenitors can be found in a small subset that is lineage negative (Lin^-), stem-cell antigen positive ($Sca1^+$), and c-Kit high, also known as the LSK subset [6]. As cells progress toward the B lineage pathway, they are transiently identified as multipotent progenitors (MPP) (which include the $Flt3^+$ lymphoid-primed multi-potent progenitor (LMPP) [7]), early lymphoid progenitor (ELP), and finally CLP. It should be noted that during these developmental stages cells maintain the ability to differentiate into other lineages. Recombination of D_H-J_H of the heavy chain locus can be detected in ELPs [8], and is actively occurring in CLPs. CLPs maintain the plasticity to differentiate into T [9-12], natural killer [13], and dendritic cells [13-16]. Although the Lin^-, IL-7Rα^+, $AA4.1^+$ (CD93), $Sca1^{low}$ CLPs [10] were originally

thought to be a branch point for multiple lymphoid lineages, they are now recognized as being B lineage specified progenitors [17]. As these cells progress to the pre-pro-B cell stage, they lose NK, DC, and T-cell potential to become committed B lineage cells.

Several reviews have discussed the interplay of transcriptional regulators during the earliest stages of B cell development that initiate B lineage specification and maintain commitment to the B lineage, maintenance of B cell identity, and elaboration of the B cell developmental program [18-21]. B cell development during the earliest stages when lineage specification is not determined is absolutely dependent upon proper regulation of B cell-specific genes.

The CLP to pre-pro-B stage

Pre-pro-B cells were originally defined by Hardy and colleagues as $CD45R^+$ $CD43^+$ $CD24^-$ cells [22]. However, it has become clear that the early stages of B cell development as defined by this FACS analysis of surface antigens represents a heterogeneous population, and exclusion of non-B lineage cells is necessary to obtain an accurate resolution of the pre-pro-B and pro-B subsets. Here I have used a FACS gating strategy based on the scheme in Li, et al [23] to identify pre-pro-B cells as Lin^- (Lin = Ter119, CD3ε, NK1.1, Ly6C, and Ly6G) IgM^- $B220^+$ $CD43^+$ $CD19^-$ $AA4.1^+$. In particular, AA4.1 has recently been identified as a marker of the earliest lymphohematopoietic progenitor in murine embryonic development and is valuable in identifying immature B lymphocytes [24].

Development from the CLP to later stages of development requires E2A proteins. *E2A* encodes the E12 and E47 transcription factors. B lymphocyte development in mice deficient in E2A proteins is blocked at the CLP stage prior to the immunoglobulin D_H to

J_H rearrangement [25]. Furthermore, E2A proteins are required for maintenance of the HSC and LMPP pool [26, 27]. The E12 and E47 proteins have differential roles during early stages of B cell development, with E47 required during earlier stages leading to the pre-pro-B stage, E12 required for later stages during recombination of the light chain locus, and both proteins playing roles in the pre-B and immature B stages [28].

The pre-pro-B to pro-B transition

Transition from the pre-pro-B to pro-B stage is accompanied with the upregulation of CD19. The CD19 co-receptor is expressed on B cells from the pro-B until the mature B cell stage, and its expression is associated with commitment to the B linage. Pro-B cells can be identified as $B220^+$ IgM^- $CD43^+$ $AA4.1^+$ $CD19^+$. Pro-B cells are actively undergoing V_H-DJ_H recombination at the heavy chain locus. If recombination leads to an in-frame coding region, a cytoplasmic Ig heavy chain can be detected within the cytoplasm, although these cells are negative for surface expression of IgM.

The early B factor (EBF) transcription factor is required for lineage specification and development from the pre-pro-B to pro-B stage. B cell development in mice lacking EBF is fully arrested at the pre-pro-B to pro-B cell transition with cells lacking D_H to J_H rearrangement [29, 30]. EBF is a primary cell fate determinant expressed solely within B lineage cells and regulates the transcription of genes required for B lineage specification. *EBF*-deficient mice lacked transcripts from *Mb-1*, *B29*, *VpreB*, *λ5*, *Rag1*, and *Rag2* as well as D_H to J_H rearrangement of the μ heavy chain [30], which are present in the early pro-B cells [31].

Once early B progenitors reach the pro-B cell stage, they are considered committed to the B lineage. However, the B cell identity and commitment must be maintained. This requires expression of B lineage genes as well as suppression of non-B lineage genes. The transcription factor Pax-5 is a secondary cell fate determinant, as it is not required for B lineage specification but is essential for B lineage commitment [21]. Pax-5 mediates expression of several B lineage-specific genes such as *Cd19*, *Mb1*, and *Blnk* and, importantly, represses non-B lineage genes such as *Notch1* [32, 33]. B cell development in mice lacking *Pax5* is blocked prior to expression of CD19 and V_H to DJ_H recombination [34]. These *Pax5*-deficient B cells express B cell-specific transcripts such as *B29*, *λ5*, and *VpreB1* and have undergone D_H to J_H recombination. However, instead of progressing along the B lineage, *Pax5*-deficient early B cells display a lack of lineage commitment and can develop into non-B lineage cells such as macrophages, dendritic cells, and natural killer cells [35].

Interestingly, progenitors that lack *E2A* proteins have reduced levels of EBF and Pax-5. EBF is also required for transcriptional activation of *Pax5*. Although the relationshihp of these early B cell transcription factors is unlikely to be simply linear, this suggests a change in the requirements for transcriptional regulators during the development and maintenance of the B lineage.

The pro-B to pre-B transition: the pre-BCR checkpoint

Pre-B cells are identified as expressing the pre-BCR on the surface and the Igμ heavy chain in their cytoplasm. This requires the cell to successfully recombine one of the two IgHC gene alleles. Only about a third of pro-B cells are able to successfully complete this process [36]. The pre-BCR is comprised of the μ heavy chain paired with

the surrogate light chain (SLC; encoded by the invariant *VpreB1/2* and *λ5* genes) (reviewed in [37]). This surface receptor associates with the Igα/Igβ heterodimer for intracellular signal transmission.

Successful signaling through the pre-BCR is absolutely critical for survival, proliferation, and developmental progression. Upon successful assembly and expression of the pre-BCR, RAG protein levels drop sharply and the cells undergo rapid proliferation [38]. In addition, *VpreB* and *λ5* transcription are silenced [39]. These rapidly proliferating pre-B cells are large as seen with FSC by FACS and are referred to as early pre-B cells.

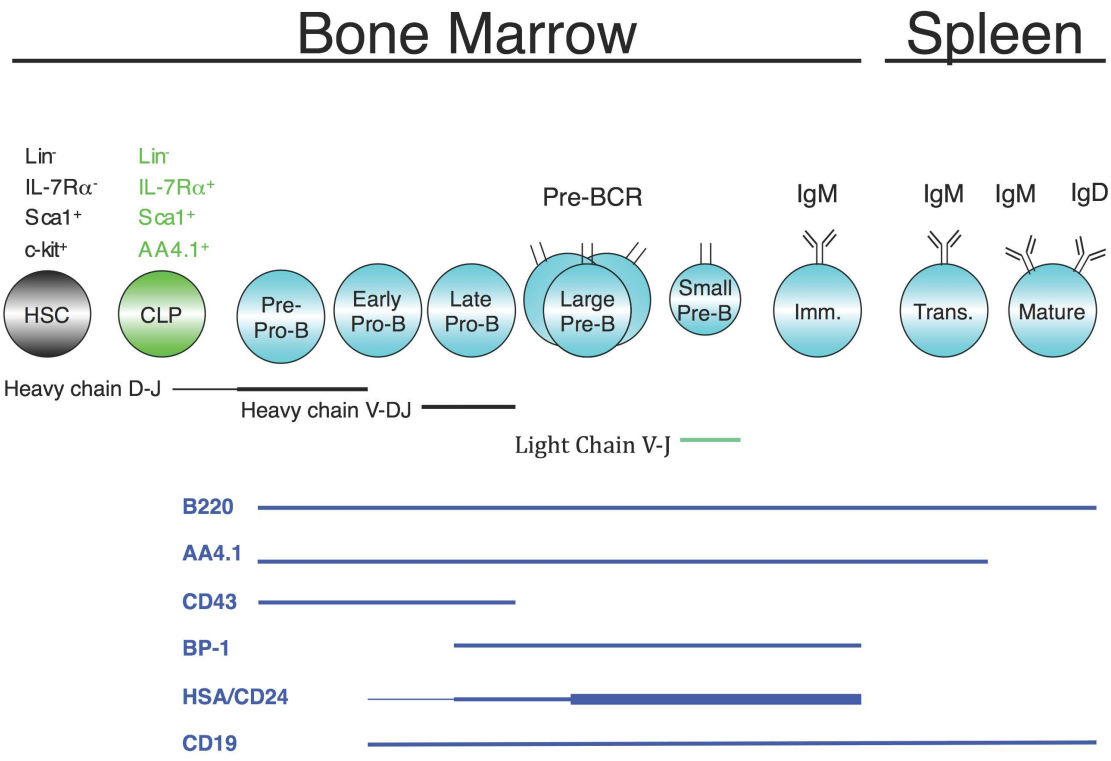
Pre-B cells expressing a pre-BCR with autoreactive potential are removed during the pre-BCR checkpoint. Selection against these potentially autoreactive Ig heavy chains requires the SLC. Mice lacking the SLC ($SLC^{-/-}$) possess increased levels of serum antinuclear antibodies (ANAs) [40].

The pre-B to immature B transition

The pre-B to immature B transition is the last developmental stage during bone marrow B lymphopoiesis. The late pre-B cell stage is associated with a cessation of proliferation and re-initiation of the recombination machinery to commence recombination of the light chain locus [38]. Light chain genes include the κ LC and λ LC. Rearrangement of the κ LC precedes that of the λ LC such that λ LC rearrangement only occurs if both κ genes are aberrantly rearranged [41]. Expression of the light chain and IgHC paired with the Igα and Igβ on the cell surface forms the B cell receptor (BCR). These cells can be identified as $B220^+$ membrane IgM^+ $AA4.1^+$. These newly

formed B cells can exit the bone marrow and travel to the spleen where additional checkpoints must be passed before a B cell can enter the long-lived mature B cell pool.

Figure 1-1. **B cell development in the adult mouse**. B cell developmental stages in the adult mouse bone marrow and spleen. Cell surface antigens used to identify specific stages are indicated. "Imm" = immature; "Trans." = transitional B cell.


Bone Marrow
Spleen
Lin-
IL-7Rα-
Sca1+
c-kit+
Lin-
IL-7Rα+
Sca1+
AA4.1+
Pre-BCR
IgM
IgM
IgM
IgD
HSC
CLP
Pre-Pro-B
Early Pro-B
Late Pro-B
Large Pre-B
Small Pre-B
Imm.
Trans.
Mature
Heavy chain D-J
Heavy chain V-DJ
Light Chain V-J
B220
AA4.1
CD43
BP-1
HSA/CD24
CD19

B cell development in the spleen

Transitional B cells

Functionally immature B cells leave the bone marrow and migrate into the periphery (Figure 1-1). Here I use the immunophenotyping strategy as described by Allman, et al [42]. Transitional B cells in the spleen are initially identified as $B220^{+}$ $AA4.1^{+}$. These can be further subdivided as IgM^{high} $CD23^{-}$ (T1), IgM^{high} $CD23^{+}$ (T2), and IgM^{low} $CD23^{+}$ (T3). These cells are considered functionally immature since crosslinking of the BCR induces apoptosis rather than proliferation [43]. Transitional B cells undergo a high rate of turnover *in vivo* as cells undergo negative selection with deletion, receptor editing, and anergy [42] (reviewed in [44]).

Mature B cells

Mature B cells can be classified as B-1 and B-2 subsets. B-1 B cells are a minor subset of mature B cells and can be subdivided into $CD5^{+}$ B-1a and $CD5^{-}$ B-1b populations based on surface phenotype and function (reviewed in [45]). B-1 B cells are located in serous cavities and spleen and respond primarily to T-cell-independent antigens. These cells are efficiently produced from fetal liver but have diminished production in adult bone marrow [46-48].

B-2 B cells represent the major population of mature B cells. B-2 B cells arise in the bone marrow and progress through multiple developmental checkpoints before emigration to the spleen where they must pass additional transitional checkpoints to become mature B cells. B-2 B cells can be subdivided into the major follicular (FO) and minor marginal zone (MZ) populations. Marginal zone B cells do not circulate and are located near the marginal sinus of the spleen where they respond to blood-borne antigens

(reviewed in [49]). Follicular B cells are located in the follicles of the spleen, recirculate through the blood, and can home to the bone marrow. These cells respond to protein antigens and, with the help of T cells, undergo class switching and affinity maturation in response to antigenic stimulation. (Peripheral B cell subsets reviewed in [50].)

Requirements for receptors in B cell development

In the following sections I highlight specific requirements for B cell development. I take an outside-in approach beginning with cell surface molecules, followed by intracellular signaling components, and ending with transcription factors. It should be kept in mind while reading this section that phenotypic analysis and markers that identify B cell subsets have changed almost as rapidly as our understanding of the developmental requirements for B cells. Therefore, what one group defines by FACS analysis as a "pre-B" cell may not be the same as a pre-B cell identified in more recent publications where a more stringent of FACS gating strategy is implemented. In the same vein, these lymphocyte "stages" are not a static group of cells but rather a continuously developing and changing population.

The antigen receptor

Perhaps the most well studied receptor on B cells, the antigen receptor is absolutely critical for B cell development. The pattern of sequential rearrangements and expression of the Ig heavy and light chains is used to define the stages of human and murine B cell development. Each receptor chain (Ig heavy chain, kappa light chain, and

lambda chain) is encoded by several different gene segments. As mentioned earlier, recombination of the receptor chain loci occurs during specific points during B cell maturation. Expression of the Igμ HC is critically required for B cell development. Mice that lack RAG proteins have no mature B or T lymphocytes [51, 52]. Failed surface expression of the antigen receptor causes an absolute block in development at specific stages, and the process of shaping the B cell repertoire weeds out B cells with poor receptor specificity and autoreactive potential during multiple stages of development.

The requirement for surface expression of the Ig heavy chain is demonstrated in mice with targeted disruption of the membrane exon of *Igμ* [53]. These mice are able to rearrange the μHC, yet the lack surface expression of Igμ. This completely blocks B cell development at the pro-B to pre-B cell transition. Thus, not only must the heavy chain be rearranged, but it must also be expressed on the cell surface for developmental progression beyond the pro-B stage.

Additional proteins interact with the μHC to form the pre-BCR. Components of the pre-BCR that make up the surrogate light chain (SLC) also show requirements during B cell development. Disruption of the *λ5* gene partially blocks B cell development at the pre-B cell stage at the transition of large pre-B to small pre-B [54]. Similar to the *λ5* knockout mouse, mice lacking both *VpreB1* and *VpreB2* are also blocked from the rapid proliferation that accompanies pre-BCR signaling [55]. In both of these mouse models, B cell development is not completely blocked, and IgM^+ B cells are found in the spleen. This suggests that components of the SLC are required for the proliferative burst that accompanies successful pre-BCR signaling but are not absolutely required for developmental progression.

In addition to the Igμ heavy chain and surrogate light chain, the pre-BCR consists of non-covalently associated transmembrane proteins Igα and Igβ that transmit intracellular signals. Deletion of Igβ completely blocks B cell development at the pro-B stage ($B220^{+}CD43^{+}$) [56]. Interestingly, these cells have undergone rearrangement of the Ig D_H and J_H segments (diversity and joining segments, respectively). However, the V_H to DJ_H recombination is compromised. This suggests that the Igβ chain may influence early pro-B cell development at a stage prior to V_H to DJ_H recombination.

For pre-B cells that successfully produce pre-BCR to develop beyond the large, cycling pre-B cell stage, they must cease dividing, undergo allelic exclusion to prevent further μHC rearrangement, and re-initiate the V(D)J recombination machinery to induce *Igκ* rearrangment [57]. Interestingly, signaling from Ras to the MEK/Erk pathway promotes B cell differentiation by driving exit from the cell cycle (through downregulating Cyclin D3, *Ccnd3*) and inducing recombination of the *Igκ* locus [58]. This differentiation to the small pre-B stage is opposed by IL-7 receptor signaling, suggesting a change in receptor requirements during early B cell differentiation processes. In support of this theory, pro-B cells cultured in low levels of IL-7 *ex vivo* yield a pre-BCR^{+} population, and expression of the pre-BCR is associated with IL-7 unresponsiveness [59]. The change in receptor requirements from the pro-B to pre-B cell stage represents an important advancement in early B cell differentiation.

Cytokines and lymphopoiesis: IL-7

Lymphopoiesis occurs in a three-dimensional space. Interaction of developing lymphocytes with their surroundings, including cell-cell contact and soluble factors, is required for proper development. Cytokines are secreted substances that initiate intracellular signaling events when bound by the appropriate cognate receptor. Cytokines with particular relevance to B cell development include interleukin-7 (IL-7) and Flt-3 ligand (reviewed in [60]). The availability of these cytokines during specific stages of B cell development adds another level of complexity to the B cell developmental process

The Fms-like tyrosine kinase-2 (Flt3 or Flk2) is a cytokine tyrosine kinase receptor that is expressed primarily during very early stages of hematopoiesis. B cell development in *Flk2*$^{-/-}$ mice is reduced only during early B cell development with decreased early B cells (examined pre-pro-B to pro-B), nearly normal numbers of pre-B cells, and normal B cell numbers from the immature stage and beyond in the bone marrow and peripheral organs [61]. Disruption of Flt3-ligand severely reduces CLP numbers in mice [62]. Flt3-ligand induces expression of IL-7Rα on hematopoietic progenitors [63], demonstrating a change in cytokine signaling as progenitors mature along the B lineage pathway. Whereas *IL-7R*$\alpha^{-/-}$ mice and *Flk2*$^{-/-}$ mice both possess some mature B cells, mice deficient in both have complete loss of mature IgM^{+} conventional B cells and visible LNs [64].

Perhaps the best-studied cytokine in early B cell development is IL-7. IL-7 is a 25 kDa type I cytokine that is secreted by resident stromal cells in the bone marrow [65] and is sufficient to induce differentiation of murine CLPs into pro-B cells under stromal-free cell culture conditions [66]. IL-7 was initially designated lymphopoietin 1

in a study to characterize the soluble factors required for precursor B cell growth in long-term stromal cell cultures [67]. Shortly thereafter, the cDNA sequence was determined, and the novel hematopoietic growth factor was renamed interleukin-7 [68]. The significance of IL-7 on B cell cultures was found to lie at earlier stages in the bone marrow (pre-B and earlier) with IL-7 having little to no effect on the proliferation of mature lymphocytes [69]. Therefore, it appears that the significance of IL-7 is found during earlier stages of B lymphopoiesis.

The IL-7 receptor is a heterodimer that is comprised of the IL-7Rα chain and the common gamma chain (γc). Expression of the IL-7Rα is associated with multiple processes during lymphocyte development, including promoting proliferation, differentiations, survival, and maintenance of the B lineage.

Disruption of IL-7 in mice demonstrates the importance of this cytokine during early B cell development. Inhibition of IL-7 receptor signaling by injection of blocking antibodies [70, 71] or genetic knockout [72] severely disrupts B cell development. Likewise, *IL-7*$^{-/-}$ mice also display impaired early B cell development [73]. B development is blocked prior to the BP1^{+} pro-B stage, and the depression of peripheral B cell numbers is not overcome with age [74]. Furthermore, administration of IL-7 to normal mice expands the numbers of immature B cells (particularly the B220^{+}BP1^{+} pro-B subset), and these numbers decline upon cessation of IL-7 treatment [75].

IL-7-deficient mice show diminished B2 subsets but maintain normal numbers of B1 and MZ B cells in the peripheral lymphocyte compartment [74]. B1 B cells represent a distinct subset from conventional B2 B cells (reviewed in [48]). In mice, B1 B cells are produced from fetal liver, and a developmental switch to B2 B cell production occurs

after birth [5]. Therefore, dependence upon IL-7 appears to be differentially required for fetal versus adult B lymphopoiesis. This is supported by studies demonstrating that HSCs from fetal liver, but not adult bone marrow, of *IL-7Rα*$^{-/-}$ mice are able to produce B cells [76]. This potential is maintained in bone marrow HSCs until one week after birth but lost by two weeks of age [76].

Although previous studies clearly demonstrate a requirement for IL-7 and its receptor during murine B cell development, a similar requirement during human B cell development has been somewhat controversial [77, 78]. Yet, current literature suggests a role for IL-7 in human B lymphopoiesis. Expression of IL-7Rα is associated with the potential of early human lymphoid progenitors to undergo clonogenic proliferation and B-lineage differentiation [79]. Recombinant human IL-7 was demonstrated to expand CD34^{+} B cell precursors from human adult and fetal bone marrow, suggesting a role for IL-7 as a growth factor during human B cell ontogeny [80, 81]. (CD34 is expressed on early B subsets, including pro-B cells, in humans [82, 83].) Furthermore, IL-7 is required for efficient survival as well as proliferation of human B cells during cell culture [84]. However, unlike murine B cells, this response is dependent upon co-culture with either human or murine stromal cells [85-87] or addition of FL3 ligand [88]. Similar to murine B cell development, dependence on IL-7 increases during ontogeny from cord blood to bone marrow in humans [81].

Signaling through the IL-7 receptor can initiate multiple signaling cascades, including PI-3K [89], Erk/MAPK [90], and activation of Stat proteins [91]. Thus, perhaps it is not surprising that IL-7 mediates multiple events in developing B cells, including proliferation, differentiation, survival, and commitment to the B lineage.

IL-7 promotes B lineage specification and differentiation

The transcription factor Pu.1 is required for expression of *IL-7Rα*, and a functional binding site for Pu.1 is located in the *IL-7Rα* promoter [92]. Retroviral expression of *IL-7Rα* can partially rescue the production of pro-B cells from *Pu.1* deficient progenitors, consistent with a regulatory hierarchy of Pu.1 and IL-7Rα [93].

IL-7 signaling contributes to B lineage commitment by promoting expression of B lineage transcription factors. CLPs from *IL-7*$^{-/-}$ mice express reduced levels of *Ebf* (early B cell factor) and *Pax5* mRNA [94]. Thus, they maintain T and NK differentiation potential but have impaired B potential. Overexpression of Ebf can partially rescue the developmental block in *IL-7Rα*$^{-/-}$ mice [94, 95]. The Ebf transcription factor is required for expression of B lineage genes such as *Pax-5*, *Mb-1* (encoding Igα), *B29* (encoding Igβ), *VpreB*, *λ1*, *Rag1,* and *Rag2* that promote B lineage specification as well as allow for differentiation [30]. *Ebf1*-deficient mice have a complete block in B cell development from the pre-pro-B to pro-B stage, similar to the IL-7-deficient mice [29].

IL-7 promotes survival and proliferation of early B subsets

During B cell development in the bone marrow, developing subsets are subjected to several selection checkpoints. This leads to a large fraction of B lineage cells being diverted toward an apoptotic pathway [96, 97]. Survival of early B subsets is dependent upon the balance of pro- and anti-apoptotic proteins within the cell (reviewed in [98]). The balance of these proteins can be influenced by the ability of the cell to respond to environmental cues (reviewed in [99]). The intrinsic (or mitochondrial or Bcl-2-regulated) apoptosis signaling pathway is directed by B-cell leukemia (Bcl)-2 family proteins. Members of this family can be grouped into three classes based on

structural and functional similarities (reviewed in [100, 101]). These include the anti-apoptosis group (Bcl-2, Bcl-xL, and Mcl-1), the pro-apoptotic group (Bax, Bak, and Bok), and the BH3-only proteins (Bad, Bid, Bim, and Puma). This pathway can be activated by growth factor deprivation in lymphocytes and lead to activation of caspases [102].

The balance of pro- and anti-apoptotic proteins influences cell survival. The pro-apoptotic proteins Bax and Bak induce permeabilization of the outer mitochondrial membrane, releasing apoptotic molecules such as cytochrome *c* (reviewed in [103]). This leads to activation of caspases, which are cysteinyl aspartate proteases that cleave substrates, activate DNases, and lead to the degradation of the cell. The anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, inhibit Bax and Bak (reviewed in [104]). BH3-only proteins can bind and regulate anti-apoptotic Bcl-2 proteins or promote the activation of Bax or Bak, leading to induction of apoptosis. Due to the opposing effects of Bcl-2 family members, a balance of the ratio of pro- and anti-apoptotic proteins is critical in determining cell survival.

Pro-B cells cultured on stromal cells express Bcl-2, and removal of pro-B cells from the stromal cell support reduces *Bcl-2* mRNA expression and elevates the levels of Bax expression, directly leading to apoptosis [105]. Apoptosis of early B subsets is enhanced in *IL-7*$^{-/-}$ mice or during culture of early B subsets from normal mice in medium lacking IL-7. This is correlated with an increased Bax/Bcl-2 ratio. Likewise, apoptosis of early B subsets is suppressed in IL-7-overexpressing mice, and this is correlated with increased Bcl-2 levels [106]. However, a direct requirement for Bcl-2 in IL-7-mediated survival is debated. Depletion of Bcl-2 with antisense oligonucelotides in the presence of

IL-7 and stromal cells does not immediately result in death of pro-B cells [105], suggesting cells are able to survive despite lowered Bcl-2 levels. In addition, transgenic overexpression of Bcl-2 rescues T cell development, but not B cell development, in *IL-7Rα*$^{-/-}$ mice [107]. Indeed, the anti-apoptotic proteins Mcl-1 and Bcl-xL have been demonstrated to restrain the pro-apoptotic activities of Bak in lymphocytes with greater affinity than Bcl-2 [108].

Loss of another anti-apoptotic protein, Mcl-1, is demonstrated to impair B cell development. Conditional deletion of *Mcl-1* with *Cd19*-Cre leads to a dramatic decrease in B cell subsets in the bone marrow [109]. Although the FACS analysis used in this study are insufficient to determine exactly where the floxed *Mcl-1* deletion occurs or the precise stage of the B cell developmental block, it is apparent that the block occurs prior to the pre-BCR checkpoint. Thus, Mcl-1 may play an essential role during early B cell development.

The requirement for pro-apoptotic proteins during early B cell survival has also been examined. Mice deficient in Bad or Bax show lymphoma and hyperplasia, respectively, yet maintain normal B cell development [110, 111]. In addition, *Bax*$^{-/-}$*IL-7Rα*$^{-/-}$ mice do not show rescued peripheral B lymphocyte numbers [112]. *Bim*$^{-/-}$ pro-B and pre-B cells show resistance to IL-7 withdrawal-induced cell death during *ex vivo* culture [113]. However, loss of Bim does not compensate for all effects of IL-7 signaling, as differentiation and proliferation of *Bim*$^{-/-}$ B220^{+}IgM^{-} cells remains dependent upon the presence of IL-7. Furthermore, the developmental block in *IL-7*$^{-/-}$ mice is not rescued in *IL-7*$^{-/-}$*Bim*$^{-/-}$ mice. However, later reconstitution studies using transfer of *Bim*$^{-/-}$ LSK cells into *IL-7*$^{-/-}$*Rag2*$^{-/-}$ recipients demonstrates that loss of Bim

allows B cell development in the absence of IL-7 [114]. This discrepancy may be due to the effects of Bim loss on the bone marrow microenvironment of *Bim*$^{-/-}$*IL-7*$^{-/-}$ mice. Another possibility is that the Oliver 2004 paper [113] analyzes cells that have developed in an IL-7-deficient microenvironment throughout fetal and adult life whereas the Huntington 2009 group [114] analyzes cells that have developed in an IL-7-sufficient environment until the LSK cells were removed from adult bone marrow and transferred into IL-7-deficient hosts.

The lymphoproliferative effects due to overexpression of IL-7 in transgenic mice are likely due to a combination of increased survival as well as proliferation. By assaying metaphase arrest as an indication of proliferative activity, pro-B and pre-B cell populations are observed to have an increased proliferative activity in IL-7 transgenic mice [115]. Often the response to IL-7 is described as "expansion," which is a misleading term, since it does not refer specifically to proliferation or cell division. For example, bone marrow from *Stat5a/5b*$^{f/-}$ mice was cultured with IL-7 to expand the lymphocyte progenitor population. These cells were then infected with a Cre-containing retrovirus (or an empty virus as control) with a GFP reporter. Four days after infection, the percentage of GFP^{+} *Stat5a/5b*$^{f/-}$ cells infected with the Cre-containing plasmid were reduced compared to those infected with the control virus [116]. This was assessed to be defective expansion of lymphocyte subsets in response to IL-7 due to loss of Stat5a/5b. However, early lymphocyte subsets naturally undergo a relatively high rate of apoptosis [96, 97]. Therefore, a disruption in proliferation or survival could both present as cell loss. Furthermore, derailment from the B lineage pathway could render a cell incapable of proliferation in an *ex vivo* environment that lacks other cytokines and growth factors

necessary for proliferation of non-B lineage cells. When determining the precise defect underlying a developmental block, it is necessary to carefully dissect the various influences on a lymphocyte population, including proliferation, differentiation, survival, and commitment to the appropriate lineage.

The GTPase Ras and the Erk/MAPK pathway

The *Ras* family of genes encodes a family of structurally related GTP-binding proteins. Mice contain three *Ras* genes, *H-Ras*, *K-Ras,* and *N-Ras*, with hematopoietic cells expressing the *N-* and *K-Ras* isoforms [117]. The status of cellular Ras activation is coordinated by GTPase activating proteins (GAPs) and guanine nucleotide-exchange factors (GEFs). GEFs activate Ras by catalyzing the exchange of GDP for GTP. GAPs stimulate the GTPase activity of Ras, leading to activation of multiple downstream pathways, including Raf and PI-3K. These pathways link Ras to multiple cellular events such as proliferation, differentiation, and survival. Dysregulation of Ras is an area of intense research due to its association with developmental disorders and cancer (reviewed in [118, 119]).

Ras has been studied during B cell development through expression of dominant negative or constitutively active Ras transgenes. Expression of a dominant-negative human *H-RAS* mutant (*H-RASN17*) in mice with the Eμ enhancer and *Lck* proximal promoter (or using the Eμ enhancer, V_H promoter, and 3' E_κ enhancer) leads to severe defects in early B cell development [120, 121]. This *H-RASN17* mutant competes for the GEF Sos, thereby inhibiting activation of all Ras isoforms. Mice expressing *H-RASN17* have a severe disruption in B cell development with a block at the pre-pro-B to pro-B

transition as well as decreased numbers of pre-pro-B cells [120, 121]. Despite the dramatic depletion of B cells in the bone marrow, splenic B cells are not as severely affected, and their number approaches littermate numbers as the mice age (despite maintaining *H-RASN17* expression). Although the precise mechanism underlying the developmental defect is not delineated, the percent of cycling pro-B and pre-B cells *in vivo* (as determined by BrdU uptake) are equivalent between *H-RASN17* transgenic mice and littermate controls. This suggests the developmental defect is not due to impaired proliferation *in vivo*.

Expression of a constitutively active Raf transgene (*Raf-CAAX*) partially rescues the developmental block in *H-RASN17* transgenic mice, although whether this is due to a partial effect of Ras signaling compensation or rather off-target effects from the constitutively active Raf transgene are not clear.

Ras has recently been linked to B cell differentiation by promoting exit of large, cycling pre-B cells from the cell cycle and re-induction of the E2A transcription factor in small pre-B cells to initiate recombination events at the *Igκ* chain locus [58]. This finding supports previous work with transgenic mouse models. First, expression of a constitutively active Ras transgene (*H-RasV12*) on a *Rag1*-deficient background allows pro-B cells to acquire characteristics of more developmentally advanced B cells, such as downregulation of CD43, expression of *Rag-2* transcripts, and population of peripheral organs [122]. In addition, constitutively active H-RasV12 drives rearrangement at the κ locus in mice lacking the J_H locus (mice lacking the J_H locus show a block in B cell development at the pro-B stage without disruption of the *Rag* genes) [123]. Together, these data implicate Ras signaling both during early B cell development at the pre-pro-B

to pro-B cell transition as well as in the differentiation of large, cycling, early pre-B cells to the small, late pre-B stage.

The adaptor protein Shc

Proper expression of cell surface receptors is necessary to engage extracellular signals required for B cell development. Likewise, the proper intracellular transmission of receptor signals through appropriate cytoplasmic signaling intermediates is critical for cells to respond to environmental cues. True adaptor molecules do not contain any enzymatic activity but rather function to recruit other proteins to signaling complexes through protein-protein interactions. In this way, they contribute to bring other molecules such as additional adaptor proteins, kinases, phosphatases, phospholipids, and GEFs into close proximity to interact during signal transduction. Adaptor proteins often contain multiple domains, allowing them to bind with specificity to various regions of other proteins and components within the cell. Additionally, adaptor proteins can often be modified by phosphorylation of tyrosine, serine, or threonine residues, creating specific docking sites for other proteins to bind only when these residues are phosphorylated. Kinase-containing molecules phosphorylate proteins on specific residues within a consensus amino acid sequence in response to stimulation, such as receptor engagement.

Due to the necessary expression of multiple receptors on early B lymphocytes, the requirements for multiple adaptor proteins have also been investigated during B cell development. Interestingly, depletion of multiple adaptor proteins has demonstrated their requirements at the pro-B to pre-B transition and beyond [124-130]. These protein-deficient mice have demonstrated multiple defects, including altered

proliferation, survival, and skewing of the ratio of mature B cell populations. However, the requirements for adaptor proteins prior to the pre-BCR checkpoint have remained elusive.

Shc structure and background

The adaptor protein Shc was identified during a search for novel SH2 sequences in a human cDNA library [131]. Three isoforms of this adaptor protein are produced via alternative translation initiation sites (p46 and p52) and alternate splice sites (p66) [132]. All three Shc isoforms contain an amino-terminal PTB domain and a carboxyl-terminal SH2 domain that are separated by a collagen homology (CH) region (denoted CH1) that is rich is proline/glycine residues (Figure 1-2). The p66 isoform contains an additional CH region (CH2) at the N-terminus.

In this manuscript, "Shc" refers specifically to the ShcA adaptor protein. After the discovery of Shc, additional family members of *Shc*-like genes were identified. These include ShcB, ShcC, and N-Shc [133-135]. Similar to Shc, these Shc-related proteins also contain the unusual combination of a PTB and SH2 domain within a single molecule and appear to function in similar intracellular signal transduction pathways such as the Ras/MAPK pathway. However, whereas Shc is ubiquitously expressed, these proteins are found only in the brain. Therefore, these additional Shc family members will not be discussed further in this manuscript.

Although Shc is a classic adaptor protein, and therefore lacks any enzymatic activity, it plays a crucial role as a bridging molecule during signal transduction. The importance of Shc is emphasized by *Shc1* knockout mice, which lack all three Shc isoforms. These mice do not survive beyond embryonic day 11.5 and show gross cardiac

defects [136]. Mice engineered to express mutated or truncated Shc protein in place of endogenous Shc also display increased embryonic lethality. Mice with a substituted Shc mutant lacking the PTB domain ($ShcA^{\delta PTB/\delta PTB}$) for endogenous Shc die by E12.5 [137]. Mice carrying a Shc mutant lacking the SH2 domain ($ShcA^{\delta SH2\delta SH2}$) or with mutation of three tyrosine residues ($ShcA^{3F/3F}$) in place of endogenous *ShcA* are born at a reduced frequency from the expected Mendelian 25% (representing 7.9% and 12.2%, respectively) and harbor defects in limb coordination [137], demonstrating the importance of specific domains and regions of Shc during embryogenesis and motor coordination.

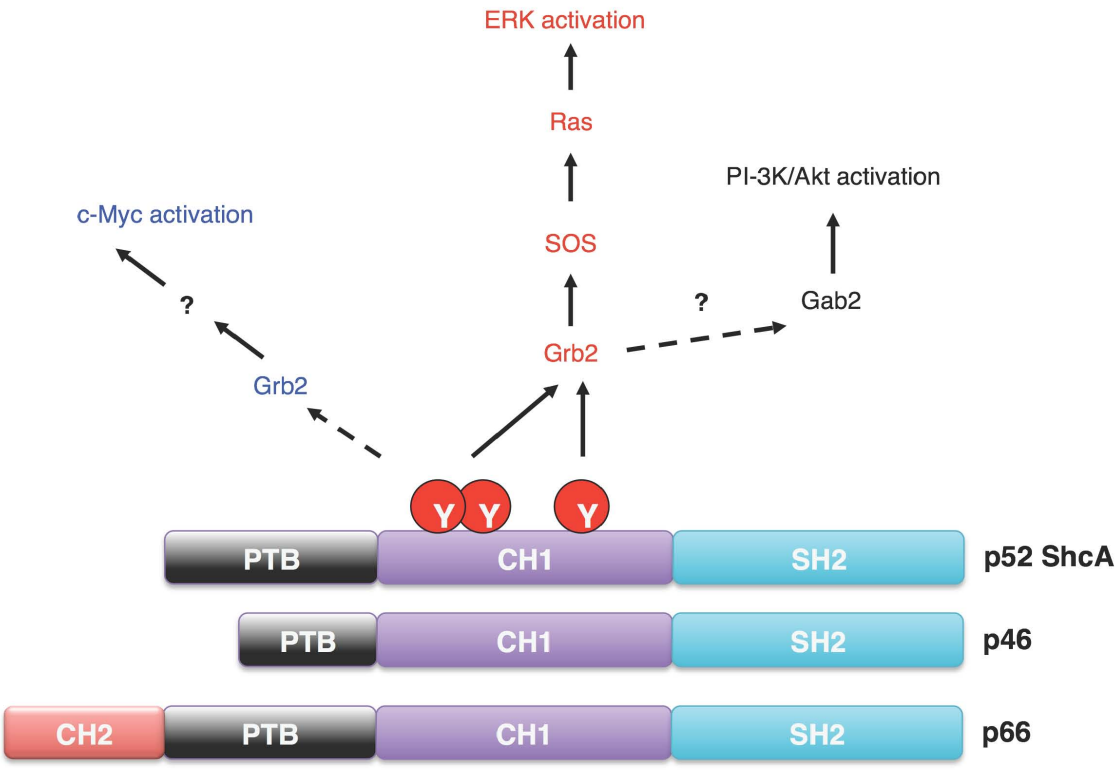
Interestingly, mice that lack only the p66 isoform of Shc show increased lifespan [138], suggesting that p66 Shc plays a different role from the p52 and p46 isoforms. Since expression of the p66 isoform is undetectable in B lymphocytes, the majority of this discussion will be with regards to p52 Shc, which is the predominate isoform in B cells.

Both the PTB and SH2 domains of Shc are capable of binding to phosphorylated tyrosine residues, although they differ in the specificity of amino acid sequences surrounding the phosphorylated residue. Amino acid specificity for PTB binding is determined by residues located N-terminal to the phosphorylated tyrosine residue whereas the specificity for SH2 binding is determined by the residues located C-terminal [139-141]. Certain receptors, such as the IL-3 receptor, can interact with both the PTB and SH2 domain of Shc, leading to its phosphorylation [142]. In addition to binding phosphorylated tyrosine residues, the Shc PTB domain can bind acidic phospholipids

[143], and the SH2 domain can bind the murine protein expressed in activated lymphocytes (mPAL) in a phosphotyrosine-independent manner [144].

The central CH1 region of Shc contains three tyrosine residues (Y239, Y240, and Y313 (mouse) or Y317 (human)) that can become phosphorylated during signal transduction events and are required for neoplastic transformation [145, 146]. This modification allows additional molecules, such as Grb2, to bind Shc and activate downstream pathways.

Figure 1-2. **Schematic view of ShcA isoform organization**. The three isoforms of ShcA are produced from use of alternative initiation sites or RNA splicing to create molecules of around 46, 52, and 66 kDa. All isoforms contain three tyrosine residues indicated in red. The Y239/Y240 and Y317 sites of p52 Shc can interact with Grb2 when phosphorylated. Phosphorylation of these residues is linked to activation of multiple downstream pathways, although not all signaling intermediates are known. Figure adapted from Ravichandran et al [147].


ERK activation
Ras
PI-3K/Akt activation
c-Myc activation
SOS
?
?
Gab2
Grb2
Grb2
Y
Y
Y
PTB
CH1
SH2
p52 ShcA
PTB
CH1
SH2
p46
CH2
PTB
CH1
SH2
p66

Shc is implicated in multiple signaling pathways

Shc is phosphorylated in response to engagement of multiple receptors. Shc phosphorylation has been linked to multiple biological outcomes, including proliferation and survival. Although the full signaling capacity of Shc is not completely illuminated, Shc is implicated in contributing to multiple pathways (reviewed in [148]) (Figure 1-2). The multiple studies to explore the signaling capacity of Shc are often performed in different cell types, and furthermore, these cell types often vary in their stages of differentiation. One should consider these variables when comparing the signaling pathways and biological outcomes resulting from Shc phosphorylation.

The Ras/MAPK pathway

The mitogen-activated protein kinase (MAPK) pathway can be stimulated by mitogens, cytokines, and growth factors. This pathway transmits signals from multiple cell surface receptors through Ras, Raf, MEK, and Erk to transcription factors in the nucleus. Signaling through the Ras/MAPK pathway can promote cellular proliferation and survival, making it an important player both during cellular development and transformation (reviewed in [149-152]).

Shc has been demonstrated to be constitutively phosphorylated in several cancerous cell lines as well as in cells transformed by v-Src or v-Fps, suggesting it might participate in mitogenic signaling pathways [131, 153, 154]. Overexpression of Shc induces a transformed phenotype in murine fibroblasts, and transformation requires the three critical tyrosine residues within the CH1 region of Shc [131, 145]. Grb2 contains an SH2 domain that allows it to bind phosphorylated Shc [155, 156]. In addition, Grb2 contains two SH3 domains that bind the GEF for Ras, Sos, leading to activation of the

Ras/MAPK pathway [157]. In support of Shc signaling to Ras, overexpression of Shc in PC12 cells results in Ras-dependent neurite outgrowth [155]. Microinjection of antibodies specific for the SH2 domain of Shc into Rat1 fibroblasts inhibited uptake of BrdU in response to stimulation with insulin, EGF, or insulin-like growth factor 1 (IGF-1) [158]. This inhibition was overcome by co-injection of oncogenic p21*Ras* (*RasT24*). These reports suggest that Ras acts downstream of Shc in conducting signaling from these growth factor receptors.

Shc phosphorylation in response to stimulation of multiple growth factor receptors (EGFR, NGFR, and IR) is implicated in activation of Ras [131, 159-161]. Using mutants of Shc further supported signaling to Ras. Expression of Shc constructs with either single or double mutations of Y239 or Y313 (the murine equivalent of Y317) impairs v-Abl-mediated proliferation and transformation of pre-B cells without affecting survival or c-Myc protein levels [162]. This is correlated with decreased phosphorylation of Raf and Erk. Conversely, expression of Shc Y239F/Y240F in Ba/F3 cells sensitized the cells to apoptosis, showing reduced *c-Myc* gene expression but normal Ras activation. Sensitivity to apoptosis is rescued with *c-Myc* expression [163]. Shc Y239F/Y240F is further demonstrated to reduce *c-Myc* expression in response to EGF-induced mitogenic stimulation [164].

The PI-3K pathway

Similar to the Ras/MAPK pathway, activation of the PI-3K pathway is also linked to multiple tumors, malignant transformation, and survival (reviewed in [165, 166]). The p85α regulatory adaptor subunit of the PI-3K contains an SH2 domain that can bind to

phosphorylated residues, such as activated growth factor receptors or the IL-7Rα chain, leading to activation of the p110 catalytic subunit [89]. In addition, the p85α subunit of PI-3K can bind phosphorylated Shc [167]. Furthermore, Shc phosphorylation contributes to activation of the PI-3K pathway in response to cytokine stimulation [168-170]. Activated PI-3K converts phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] into phosphatidylinositol-3,4,5-triphosphate [$PI(3,4,5)P_3$], which recruits PH-containing molecules, such as Akt, to the plasma membrane (reviewed in [171]).

Mice deficient for *Pi85α* show impaired B cell development beginning near the pro-B cell stage [172]. These mice also harbor an altered splenic B cell compartment with decreased transitional, follicular, and marginal zone B cell numbers [128]. Mature B cells show impaired response to anti-IgM and LPS stimulation with decreased proliferation and increased apoptosis [173]. This suggests an important role for PI-3K in conducting receptor signals during B cell development, although the precise stage where PI-3K is required has not been clearly established.

Brief summary of the following chapters

In the next chapter, I will detail the experimental procedures used in the current study. The following two chapters will focus on the experiments and results that have led me to determine that Shc and Shc phosphorylation are important for early B cell development. In chapter 3, I describe the animal models that were crucial for selective testing of Shc in B lymphocytes as well as analyses to determine the exact stage at which B cell development is perturbed in mice with disrupted Shc signaling. I utilize conditional dominant negative ShcFFF transgenic mice and conditional *Shc* knockout

mice that were previously utilized to identify a requirement for Shc in T cell development [174]. In chapter 4, I investigate potential mechansims of the developmental defect. Decreased cell numbers in the pro-B cell pool can be due to multiple defects, including developmental progression from the pre-pro-B stage, proliferation, commitment of the pro-B cells to the B lineage, and survival. Chapter 5 will summarize my findings and suggest future directions to further our understanding of Shc in B lymphocyte development. In addition, I have included in Appendix I preliminary data from a project that may provide an interesting future direction with the interaction of phosphorylated Shc with the c-Abl tyrosine kinase. Together, these data provide a novel role for Shc during B cell development with a significant implication in IL-7 signaling. Findings from this study were published in [175.].

Chapter II

Materials and Methods

Mice

The conditional ShcFFF transgenic mouse line and conditional *Shc1* knockout have been described previously [174]. The *Mb1*-Cre and *Cd19*-Cre mice have also been previously described [176-178]. The Bcl-2 transgenic mice (C57BL/6-Tg(BCL2)36Wehi/J) have been previously described [179]. Rag-1-deficient mice were obtained from Jackson Laboratories (Bar Harbor, Maine). The LSL K-ras G12D mouse (strain B6.129-Kras^{tm4Tyj}) was obtained from Mouse Models of Human Cancers Consortium (MMHCC) [180]. Mice were bred and maintained under specific pathogen-free conditions at the University of Virginia animal facility according to approved IACUC protocols.

Mouse genotyping

Mouse tail DNA as well as sorted cell populations were used as template for PCR reactions. Tail DNA was digested overnight in DirectPCR lysis reagent (Viagen) with 0.25 μg/μL Proteinase K. Digested tails were heat-inactivated at 70°C for 1 hour, and the crude lysates were used for PCR templates. Sorted cells were lysed in lysis buffer (100 mM Tris.HCl pH 8.5, 5.0 mM EDTA, 0.2% (w/v) SDS, 200 mM NaCl, 0.25 μg/μL Proteinase K) and then purified with isopropanol precipitation. After rinsing with ice-cold 70% ethanol, DNA was resuspended in dH_2O and used as template for PCR.

Primers were used as 1 μL of 10 mM primer per 25 μL PCR reaction. All primers are listed 5' to 3'.

ShcFFF: The following primers can be used in the same PCR reaction to create the following bands: STOP deleted, 833 bp, 2106/EF1 + 4329/Shctg; Nonrecombined ShcFFF transgene, 511 bp, 2106/EF1 + 2617/STOP. 2106/EF1: TGAGTCACCCACACAAAGGA (binds 5' to the first *loxP* site of ShcFFF; a forward primer), 2617/STOP: TTCTTCGGTGGAAACAACGG (binds 3' to the first *loxP* site; a reverse primer), 4329/Shctg: TGGAGACGGTGAGAGTGATT (binds 3' to the second *loxP* site; a reverse primer).

Floxed Shc1: The following primers can be used in the same PCR reaction to create the following bands: Wild-type *Shc1*, 302 bp; floxed *Shc1*, 350 bp; deleted *Shc1*, 600 bp. 3338: CAGCCGGCCAACTCTAAG; 3640: GCCCTCGGACAGAGCAATCATGTC; 8388: GTCAAGGGCTCGCATTGACTG.

Cd19-Cre: This PCR protocol allows the detection of both the endogenous *Cd19* locus as well as the *Cd19*-Cre inserted locus. Thus, it can be used to distinguish heterozygous from homozygous *Cd19*-Cre mice. The following primers can be used in the same PCR reaction to create the following bands: wild-type *Cd19*, 450 bp; *Cd19*-Cre, 750 bp. Cre7: TCAGCTACACCAGAGACGG; CD19C: AACCAGTCAACACCCTTCC; CD19D: CCAGACTAGATACAGACCAG.

Mb1-Cre: The following primers allow detection of *Mb1*-Cre as well as the endogenous *Mb1* locus. I do not perform this as one reaction. These PCR reactions allow me to distinguish between homozygous and heterozygous *Mb1*-Cre mice. Cre primers are not specific for the *Mb1*-Cre and will detect any iCre construct. Homozygous *Mb1*-Cre mice are extremely useful as breeders if bred to a Cre negative mouse. However, it should be noted the homozygous *Mb1*-Cre mice tend to become sick and die earlier than heterozygous *Mb1*-Cre. The following primers will produce a 430 bp product if *Mb1*-Cre is present. hCre dir: CCCTGTGGATGCCACCTC; hCre rev: GTCCTGGCATCTGTCAGAG. The following primers will produce a 400 bp product if the wild-type (no Cre) *Mb1* locus is present: mb1 probe dir: CTGCGGGTAGAAGGGGGT; mb2 in2rev: CCTTGCGAGGTCAGGGAGCC.

Rag1: The following primers allow detection of *Rag1* or deletion of *Rag1* in one PCR reaction. Expected results are as follows: wild-type: 474 bp, disrupted *Rag1*: 530 bp. oIMR1746: GAGGTTCCGCTACGACTCTG; oIMR3104: CCGGACAAGTTTTTCATCGT; oIMR8162: TGGATGTGGAATGTGTGCGAG.

Bcl2 transgenic mice: The following primers allow detection of the Bcl-2 transgene. Presence of the Bcl-2 transgene will yield a 150 bp product. oIMR550: TGGATCCAGGATAACGGAGG; oIMR551: TGTTGACTTCACTTGTGGCC.

KrasG12D transgenic mice: The following primers allow detection of the kRasG12D transgene. Primers K004 and K006 are used as an internal control to confirm the

presence of amplifiable DNA. Primer K005 is specific for the 3' end of the LSL element, while primer K004 is specific for Kras2 exon 1. The following should result from a PCR reaction with K004/K006: 500bp and K005/K006: 550 bp (and indicates presenece of STOP cassette). K004: GTCGACAAGCTCATGCGGGTG;
K006: CCTTTACAAGCGCACGCAGACTGTAGA;
K005: AGCTAGCCACCATGGCTTGAGTAAGTCTGC.

Southern blotting

Genomic DNA was prepared by overnight lysis in 500 μL lysis buffer, and DNA was purified with isopropanol precipitation. Precipitated DNA was washed in ice-cold 70% ethanol and resuspended in dH_2O. DNA was digested 4 hours to overnight and separated on a 1% agarose gel. After electrophoresis, agarose gels were incubated in 0.25 M HCl for 15 minutes followed by a 30 minute incubation in 0.4 M NaOH. DNA fragments were transferred to a GeneScreen Plus membrane (NEN Life Science Products) by standard alkaline transfer overnight. Pre-hybridization with salmon sperm DNA was performed for 1 hour at 65°C (pre-hybridization buffer: 5 M NaCl, 1 M Tris pH 7.5, 10% (w/v) SDS, 10g Dextran Sulfate/100mL, and 2.5 mg/mL salmon sperm DNA). The radioactive probe was created with Shc cDNA as template and labeling with α^{32}P-dCTP using the random primer DNA labeling kit (TaKaRa Co.). Free primer was removed using QIAquick nucleotide removal kit (Qiagen). The denatured probe was added directly to the membrane which was incubating in pre-hybridization buffer and allowed to incubate overnight at 65°C. Membrane was washed twice with 65°C wash

buffer (1xSSC, 0.1% (w/v) SDS) for 20 minutes at 65°C. Radioactivity was detected by autoradiography at -80°C.

Flow cytometry

Single-cell suspensions were prepared from bone marrow and spleen of 8-12 week-old mice. Red blood cells were lysed, and cells were stained for FACS analysis in 0.5% BSA in PBS with 0.05% NaN_3. Antibodies specific for the following epitopes were used: B220 (RA3-6B2), CD19 (1D3), CD93 (AA4.1), BP-1 (6C3), CD24 (30-F1), TER119, CD11b (M1/70), CD3ε (145-2C11), IgM (II/41), Ly-6G (RB6-8C5), CD43 (S7), CD127 (A7R34), CD117 (2B8), Sca1 (D7), and BrdU. Annexin V-FITC was used to detect surface exposure of phosphatidyl serine. Antibodies were conjugated to FITC, Alexa488, PE, PE-Texas Red, PE-Cy5.5, PE-Cy7, V450, APC, Alexa647, APC-Alexa750, or APC-eFluor780. Biotin-conjugated antibodies were detected using streptavidin-FITC, PE, APC, or Pacific Orange. Cell viability or DNA content was analyzed with 4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI), 7-Amino-actinomycin D (7-AAD), or propidium iodide (PI). Antibodies and other reagents were purchased from ebiosciences, BD PharMingen, or Sigma-Aldrich. FACS data was collected at the University of Virginia Flow Cytometry Core Facility on a CyAn ADP 9 color machine. FACS analysis was performed using FlowJo software and gating on "singlets" as determined by pulse width versus forward scatter.

For detection of phosphorylated Erk by flow cytometry, I followed the protocol outlined in Chow, et al [181]. The E10 antibody for phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) was used. This antibody was conjugated to Alex Fluor 647.

In vivo BrdU incorporation assay

To observe cell cycle kinetics, I utilized the protocol outlined by Kincade, et al [182]. For bone marrow analysis, mice received an initial 0.1mg BrdU (Sigma-Aldrich) in 200μL PBS injected intraperitoneally (IP) followed by continuous BrdU administration in water (0.8mg/mL; changed daily). A minimum of five control and five mutant mice were sacrificed at 24, 48, and 72 hours post-injection. Bone marrow from two femurs per mouse was harvested, RBC lysed, counted (by trypan exclusion), and stained for surface phenotype. BrdU incorporation was determined by flow cytometry using a BrdU incorporation kit (Becton Dickinson). The least squares analysis was performed to obtain the renewal and production rates.

Ex vivo proliferation assays

Bone marrow B cells were sorted at the University of Virginia Flow Cytometry Core Facility with a Becton Dickinson FACSVantage SE Turbo Sorter with DIVA Option. Sorted cells were collected into fetal bovine serum, rinsed in 1x PBS, and plated at 7,000 cells per well in triplicate in a 96-well plate. For mice on a *Rag1*$^{-/-}$ background, pro-B cells were collected using MACS anti-CD19 microbeads (Miltenyi) per the manufacturer's protocol and assessed to be >95% pure by FACS analysis of anti-B220 by anti-CD19. Pro-B cells were grown for three days in the indicated concentration of IL-7 (Pepro Tech) in 100μL RPMI 1640 with 5% FCS, 2mM L-glutamine, and 50μM β-mercaptoethanol (complete RPMI) while incubating at 37°C in a humidified chamber with a 5% CO_2 atmosphere. On the third day, 50μL of fresh complete RPMI with the indicated concentration of IL-7 were added to feed the cells. During long-term cultures

on OP-9 stromal cells, cultures were fed every three days. For [^{3}H]-thymidine uptake assays, 1μCi of [^{3}H]-thymidine was added to each well 6-8 hours before harvesting. Cells were collected on Filtermat A filters (PerkinElmer Life Sciences). For total cell counts, 100μL of cell suspension were added to a FACS tube along with 50μL of 5μm counting beads (SpheroTech) and PI for viability. Live cells were gated using PI versus FSC, and total live cell number was calculated per the manufacturer's protocol. Remaining cell suspension was used to phenotype B cell subsets by FACS analysis.

Cell culture and transfection

Cells were maintained in a humidified 37°C incubator with 5% CO_2. 293T cells were maintained in Dulbecco's Modification of Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin/glutamine (PSG). K562 cell lines were maintained in Iscove's Modification of DMEM (IMDM) with 10% (v/v) FBS and 1% (v/v) PSG. For long-term storage, cells were resuspended at 5×10^6 cells/mL in FBS with 10% (v/v) DMSO. 1mL of cells were aliquoted to freezer tubes and frozen at -80°C in slow-freeze containers. Cells were then transferred to liquid nitrogen for long-term storage. Primary lymphocytes were cultured in RPMI-1640 medium with 5% (v/v) FCS, 1% (v/v) PSG, and 50 μM beta mercaptoethanol (BME). 293T cells were transiently transfected with the calcium phosphate method.

Cell activation, immunoprecipitation, and immunoblotting

Pro-B cells from *Rag1*$^{-/-}$ bone marrow were collected using anti-CD19 microbeads (Miltenyi) per the manufacturer's protocol. Cells were allowed to rest in plain RPMI in a 37°C humidified chamber with a 5% CO_2 atmosphere for one hour before stimulation. Cells were then transferred to Eppendorf tubes in a 37°C water bath for stimulation. Cells received 100ng/mL IL-7, removed at the indicated times, and immediately boiled in 1x Laemmli buffer with 2% β-mercaptoethanol. "Unstimulated" cells received PBS alone and were incubated for 5 minutes. Lysates were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Phosphorylated Shc was detected with anti-phospho-Shc antibody (pY239/pY240, Santa-Cruz). Total Shc protein was detected with rabbit polyclonal antibody (BD Biosciences).

For detection of FLAG-tagged ShcFFF transgenic protein, B cells from bone marrow or spleen were positively selected using anti-CD19 microbeads (Miltenyi). Cells were rinsed with PBS and lysed. Total protein from cleared lysates was determined with Bradford Reagent. Equal protein levels between control ($Cre^{-}ShcFFF^{+}$) and mutant ($Cre^{+}ShcFFF^{+}$) were subjected to immunoprecipitation by anti-FLAG agarose (Sigma Chemical Co.). Immunoprecipitates were rinsed 4x with 500μL cold lysis buffer and immunoblotted as described above. For detection of endogenous Shc protein in the conditional *Shc1* knockout mice, total bone marrow B cells were first enriched with anti-CD19 microbeads (Miltenyi) and then sorted as described above. Total cell lysates were probed for total Shc and Erk1/2 protein as a loading control.

Migration to SDF-1α

$CD19^+$ B cells were enriched from bone marrow with anti-CD19 microbeads (Miltenyi). Cells were resuspended at 2×10^6 cells/mL in RPMI with 0.5% BSA (migration medium) and allowed to rest in a 37°C humidified incubator while transwells were assembled. Transwells with 8 μm pore size were used for migration analyses. Migration medium with or without varying concentrations of SDF-1α was placed in the bottom well, and 2×10^5 cells were placed in the upper well. Cells were allowed to migrate for 2 hours in the 37°C incubator. Remaining input cells were maintained in migration medium in the incubator. After the migration period, the Cell Titer Glo (Promega) assay was used to quantify viable cells present in the bottom well of each transwell as well as the input cells by quantifying ATP levels. FACS analysis of the remaining migrated cells (and input cells) was also performed.

Calcium flux assay

Splenic B cells were collected with anti-CD19 microbeads (Miltenyi) and incubated in fluorimetry medium with 1 μg/mL Indo-1 (Molecular Probes, Eugene, OR) in a 37°C incubator for 20 minutes. Fluorimetry medium consists of 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes pH 7.4, 0.1% glucose, and 1% fetal calf serum. Cells were then washed twice with fluorimetry medium, resuspended in fluorimetry medium, and used for stimulation. The ratio of Indo-1 fluorescence at 398 and 480 nm was recorded using a Hitachi Model 2500 fluorescence spectrometer. The background was recorded for 20 seconds, followed by addition of either goat anti-mouse IgM (Jackson ImmunoResearch) or rabbit anti-mouse IgM

(Zymed; now Invitrogen, after dialysis against PBS to remove sodium azide). Anti-IgM antibodies were used as either intact (for stimulation of the BCR and FcγRIIB (FcR)) or with only the $F(ab')_2$ fragment (for stimulation of the BCR alone).

Plasmids and mutagenesis

All constructs generated were sequenced to confirm the sequence fidelity and presence of appropriate mutations. All constructs were transformed into DH5α *E. coli* bacteria and frozen in glycerol stocks after confirming insert fidelity through DNA sequencing.

ShcCH-Lck

The CH1 region of SHC and kinase domain of Lck were cloned by PCR. The following plasmids were propagated from frozen bacterial stocks and used for template: ShcCH: ShcCH:208-377 wt pEBG; $ShcCH^{FFF}$: ShcCH:208-377 Y239F Y230F Y317F pEBG #2; $ShcCH^{YYF}$: ShcCH YYF 208-377/pGEX2T #7; $ShcCH^{FFY}$: ShcCH FFY 208-377/pGEX2T #13; Lck kinase domain: Lck-wt/pEF. PCR primers for the ShcCH and ShcCH mutants created EcoRI and NdeI restriction sites with the 5' primer and SpeI and BamHI restriction sites with the 3' primer. The NdeI site created a 5' ATG sequence for the ShcCH region as well as an additional restriction site. Primers for the Lck kinase domain created EcoRI and SpeI restriction sites with the 5' primer and a PstI restriction site with the 3' primer. The PCR product for the LCK kinase domain encompassed the sequence encoding amino acids 226-509 of human LCK kinase. The resulting PCR products for ShcCH, $ShcCH^{YYF}$, $ShcCH^{FFY}$, $ShcCH^{FFF}$, and LCK were cloned each

restricted and placed into a separate pGBT9 vector. ShcCH and ShcCH^{FFF} were inserted with EcoRI and BamHI into pGBT9 vector that had been restricted with the same enzymes. After restriction, the pGBT9 restriction digest was run on a 1% agarose gel to separate the plasmid from insert. The restricted pGBT9 plasmid was then excised and purified (Quiagen). PCR products were also purified after restriction digestion (Quiagen). The pGBT9 vector contains the Gal4 DNA binding domain, and the inserted ShcCH and ShcCH^{FFF} were kept in-frame with the Gal4 DNA binding domain. LCK was inserted into the pGBT9 after restricting the LCK PCR product and pGBT9 vector with EcoRI and PstI. To create the ShcCH-LCK and ShcCH^{FFF}-LCK fusion proteins, ShcCH.pGBT9, ShcCH^{FFF}.pGBT9, and LCK.pGBT9 were all restricted with PstI and SpeI and gel-purified. The purified LCK fragment was then sub-cloned into ShcCH.pGBT9 and ShcCH^{FFF}-Lck to create ShcCH-Lck.pGBT9 and ShcCH^{FFF}-Lck.pGBT9. The pGBT9 yeast bait vector fuses the Gal4 DNA binding domain to the N-terminus of the bait protein (ShcCH-LCK fusion proteins) and rescues the Trp auxotrophy.

Grb2SH2

The SH2 domain of Grb2 was cloned by PCR. Bacteria containing the Grb2SH2.pGEX plasmid were grown from bacterial stocks, and the Grb2SH2.pGEX plasmid was collected by miniprep (QIA prep) and used for template. PCR primers for Grb2SH2 introduced a BamHI restriction site with the 5' primer and a NotI site with the 3' primer. The Grb2SH2 PCR product was then inserted into the pVP16 vector after restricting PCR product and target vector with BamHI and NotI. This created

Grb2SH2.pVP16. The pVP16 yeast prey vector fuses the Gal4 activation domain to the N-terminus of the prey protein (Grb2SH2) and carries the Leu selectable marker.

c-Abl constructs

Murine c-Abl (I) received from Open Biosciences (accession #: BC059260, clone ID: 6408990) in pYX-Asc vector (Amp resistant; sequencing primers: T3, T7; 5' restriction site: EcoRI; 3' restriction site: NdeI). Murine c-Abl constructs also received from Jean Wang: c-Abl (IV)(FLAG).pMSCV(hph) and c-AblμNLS(IV)(FLAG).pMSCV(hph)

Yeast two-hybrid

The HF7c yeast strain was used for the yeast two-hybrid experiments. This strain is auxotrophic for His, Trp, and Leu. The principle of this system as used here is the bait vector, pGBT9, rescues the Trp auxotrophy and encodes a protein fused to the Gal4 DNA binding domain (DNA-BD). The prey vector, pVP16, rescues the Leu auxotrophy and encodes a protein fused to the Gal4 activation domain (AD). If the bait and prey proteins interact, this permits drives production of His, and yeast can grown in medium lacking the essential amino acids His, Trp, and Leu.

Yeast transformation

Yeast were transformed using the lithium acetate transformation protocol. Overnight cultures of HF7c were collected by centrifugation, rinsed with water, and resuspended in 0.1M lithium acetate. Yeast were added to Eppendorf tubes containing

salmon sperm carrier DNA as well as the plasmid of interest. After brief vortexing, 40% w/v PEG/0.1M LiOAc were added to each tube. Tubes were briefly vortexed and incubated at 30°C for 30 minutes with shaking. DMSO was then added to each tube, and the mixtures were mixed by gentle inversion. Cells were heat-shocked at 42°C for 15 minutes then chilled on ice for two minutes. Cells were pelleted, resuspended in water, and plated onto selective medium plates. For the yeast two-hybrid assay, yeast were first transformed with ShcCH-Lck.pGBT9. After growth on selective medium (SD-Leu), yeast were subsequently transformed with a seven-day mouse embryo library and plated onto SD-LW plates. Yeast were allowed to grow on SD-LW plates and then replica-plated onto SD-LWH plates with 10mM 3-amino-1,2,4-triazole (3-AT) to select for transformants that possessed interacting bait and prey proteins. The 3-AT is a competitive inhibitor of the product from the HIS3 gene. This helps to ensure that only proteins that interact strongly enough will be able to produce enough His to grow on the selective plates. Colonies growing on SD-LWH + 10mM 3-AT were streaked onto SD-LW plates. Yeast that grew after streaking were collected to identify prey sequences.

Collection of positive prey plasmids

Colonies from the yeast two-hybrid assay were grown overnight in 5mL SD-LW medium. Cells were pelleted, resuspended in 250 μL P1 buffer, and transferred to Eppendorf tubes. To this tube, 250 μL P2 buffer were added and tubes inverted 6-8 times. To this, 350 μL N3 buffer were added and tubes inverted 6-8 times. Lysed cells were pelleted, and supernatants containing plasmid DNA were placed onto Miniprep columns. Columns were centrifuged, rinsed with PE buffer, and plasmid DNA were

eluted with 25 μL EB buffer. All buffers were from QIA kits. Prey plasmids were transformed into KC8 *E. coli* (10 μL eluted DNA to 100 μL thawed cells). Bacteria and DNA were transferred into 2 mm gap electroporation tubes and electroporated (25 μFD, 200 Ω, and 2.5 KV with time constant of about 4.6 msec). Electroporated cells were transferred to Eppendorf tubes, resuspended in SD-LW broth, and plated on M9-Leu plates.

Chapter III

Disruption of Shc signaling impairs early B cell development

Although this was not one of my initial projects upon joining the laboratory, it evolved into my primary project. Findings from this study were published in [175]. Previous studies in our laboratory demonstrated that Shc was required for T cell development by contributing to signal transduction pathways downstream of the pre-T cell receptor. This impaired T cell development by inhibiting the proliferative burst that accompanies successful pre-T cell receptor expression and signal transduction. Due to the similarities between T and B cell development, I hypothesized that Shc would play a similar role in B cell development at the homologous pre-B cell receptor checkpoint by transmitting signals from the pre-B cell receptor that lead to proliferation at this checkpoint. What truly intrigued me with this project was that my initial hypothesis was wrong, and I have pursued the ensuing research to learn the correct answer to my broader question: does Shc play a role in B cell development?

Abstract

The adaptor protein Shc is phosphorylated on three critical tyrosine residues in response to stimulation of multiple cell surface receptors, including antigen and cytokine receptors. Successful expression and signal transduction from cell surface receptors is essential during the highly ordered process of B cell development. However, the

requirement for Shc in B cell development has not been addressed. In this chapter I utilized conditional mouse models to address the requirement for both Shc protein and its three critical tyrosine residues during B cell development during either early or later stages of B cell development. I found that both expression of Shc protein and its ability to become tyrosine phosphorylated are required during early B cell development. Specifically, Shc is required for production of proper numbers of pro-B cells. This developmental stage is earlier than the pre-T cell receptor checkpoint, which is where Shc was shown to be required during T cell development. Therefore, Shc is required at an earlier stage in B cell development than in the similar T cell development.

Introduction

Shc is an adaptor protein that has been shown to signal downstream of multiple cell surface receptors. A well-studied mechanism of Shc signaling is the phosphorylation of three critical tyrosine residues within the CH1 region of Shc in response to receptor stimulation. Shc is demonstrated to become tyrosine phosphorylated in response to stimulation of multiple receptors, including antigen receptors (TCR [183], BCR [184]), cytokine receptors (CXCR4 [185], IL-2 [186], IL-3 [187, 188], IL-9 [189]), and growth factor receptors (PDGFR [190], EGFR [131], insulin receptor [159], and NGFR [155]). Shc is also phosphorylated in cells expressing BCR-ABL and plays a role in the efficacy of BCR-ABL-mediated hematopoietic transformation [191, 192]. Shc phosphorylation is also correlated with tumorigenicity owing to the observation that it is often constitutively phosphorylated in tumor cells [154]. Furthermore, signaling from phosphorylated Shc is linked to breast cancer development and poor prognosis (reviewed in [193]).

The process of lymphopoiesis represents a highly ordered and selective series of events in which developing cells must survive, proliferate, and differentiate in order to progress from a HSC to a mature, functional lymphocyte. During this process, lymphocytes must competently express cell surface receptors and properly transmit intracellular signaling events in response to receptor stimulation. Disruption of this process by loss of receptor expression or the functional competence of intracellular signaling intermediates can have severe consequences on lymphopoiesis and halt lymphocyte production at specific stages of development. Due to the known involvement of Shc in signaling from multiple cell surface receptors and the stringent requirement for multiple receptors in lymphocyte development, it seemed likely that Shc could play a role

in B lymphocyte development by conducting intracellular signals from cell surface receptors.

Previous work in B cell lines focused on the role for Shc in response to antigen receptor stimulation in IgM^+ B lymphocytes. Shc was suggested to be phosphorylated by the protein tyrosine kinase Syk in response to BCR stimulation [194]. This creates two Grb2 binding sites, which were hypothesized to bind Grb2/Sos and lead to activation of the Ras/MAPK pathway [195]. However, this hypothesis was challenged in the immature B cell line DT40. Disruption of *Grb2* but not *Shc* impaired ERK activation in response to BCR stimulation [196]. This suggested that Shc was not a dominant player in BCR signaling to ERK activation. However, this finding did not rule out a requirement for Shc during B cell development by conducting signals downstream from other cell surface receptors (perhaps even leading to ERK activation). Furthermore, this did not rule out a role for Shc in signaling downstream of the BCR, as the cell line tested represented only one stage of B cell development. The requirement for certain signaling players can change as a cell differentiates and matures. Finally, this paper only addressed a potential positive role of Shc on ERK activation. Indeed, later papers found a potential role for Shc in *negative* signaling downstream of the Fc receptor by forming a complex with the inositol phosphatase SHIP (reviewed in [197]).

As discussed below in this chapter I tested the requirement for Shc during B cell development by using mice designed to conditionally disrupt Shc signaling during different stages of B cell development via the Cre/*loxP* approach. I found that targeted expression of a dominant negative Shc protein or selective deletion of germline *Shc1* transcription severely blocked B cell development but only when the disruption occurred

during early developmental stages. As I had hypothesized based upon previous experiments demonstrating a requirement for Shc at the pre-TCR checkpoint, I observed decreased pre-B cell numbers in mice with disruption of Shc signaling. However, I also noticed a surprising defect in the cellularity of the pro-B cell population. Upon further analysis, I identified a block at the pre-pro-B to pro-B transition, which is prior to any defects related to pre-BCR signaling. This suggested that Shc and Shc phosphorylation played a previously unknown role during B cell development, and unlike T cell development, the requirement for Shc was placed prior to the pre-BCR checkpoint. Here, and throughout this dissertation, I use "transition" to refer to a change in developmental pools, which does not discriminate between developmental progression, proliferation, survival, or maintenance of the B lineage. Rather, these individual defects were addressed to idenfiy the cause of the developmental block in later chapters.

Results

Targeted disruption of Shc during B cell development

To determine the requirement for Shc during early B cell development, I utilized mice that had been previously generated in our lab for targeted disruption of Shc [174]. First, I used mice with targeted expression of a transgene encoding a dominant negative FLAG-tagged human Shc protein with mutation of its three critical tyrosine residues (Shc Y239F/Y240F/Y317F; hereinafter ShcFFF) (Figure 3-1). Human p52 Shc shares 94% amino acid identity with mouse p52 Shc, including the three critical tyrosine residues in the CH1 region (Figure 3-2). Many previous studies have used human and mouse p52 Shc interchangeably. Therefore, it was reasonable to assume that the human p52 ShcFFF transgene likely behaved similarly to the murine p52 Shc in all capacities aside from interacting with proteins that would normally bind the phosphorylated tyrosine residues that are mutated in p52 ShcFFF.

The second model employed to disrupt Shc signaling was conditional *Shc1* knockout mice [174]. In these mice, the first and second exons of *Shc1* are floxed with *loxP* sites (Figure 3-3). The floxed genomic sequence encodes the transcriptional start sites and amino termini of all three Shc isoforms. Cre-mediated recombination at the *loxP* sites halts production of all three Shc isoforms of ShcA.

There is a phenotype associated with mice in which the *Shc1* locus is floxed. These mice are smaller than their littermates that have one or both wild-type (non-floxed) *Shc1* loci. Unpublished observations by Gronski, et al in our laboratory (manuscript submitted), demonstrate that the *loxP* site located upstream of the *Shc1* locus is inadvertently inserted into the first exon of *Cks1* (Cdc kinase subunit 1; with

transcriptional direction opposite that of *Shc1*), disrupting its expression (Figure 3-3). For this manuscript I compared mice that were *Shc1*$^{f/f}$ to littermates that retained one or more wild-type *Shc1* alleles as well as mice deficient in *Cks1* (mice obtained from Dr. Steve Reed). My analyses did not reveal any difference in the percentages of B cells in any developmental subset in the bone marrow or spleen. Thus, it is unlikely that disruption of *Cks1* contributed to any phenotype in the developing B cell subsets of floxed *Shc1* mice.

To target conditional expression of the ShcFFF transgene or conditionally delete *Shc1* in developing B lymphocytes, I utilized two Cre transgenic lines to direct Cre-mediated recombination at different stages of B cell development.

To target the early B cell developmental stages, I used the Cre transgenic line *Mb1*-Cre. *Mb1*-driven Cre-mediated recombination can be detected in the pre-pro-B stage and continues throughout B cell development, allowing strong expression of Cre recombinase during early B development (Figure 3-4) [176, 177, 198]. In *Mb1*-Cre/ShcFFF mice the ShcFFF protein was detected by anti-FLAG immunoprecipitation of CD19^{+} B cells from both the bone marrow and spleen, consistent with the expected Cre expression pattern (Figure 3-5). *Cd19*-Cre expression is reported to begin weakly at the pro-B stage, but this Cre strain was largely beneficial for examining the role of Shc during later B cell development (Figure 3-4) [178]. Although the reported expression of *Cd19*-Cre suggests weak expression beginning at the pro-B stage, I was only able to detect the ShcFFF protein in CD19+ cells from the spleen of *Cd19*-Cre/ShcFFF mice (Figure 3-5). Since these mice only expressed detectable levels

of ShcFFF in splenic B cells, I used them as a model to assess the requirement of Shc during later stages of B development.

Figure 3-1. **Schematic of conditional Shc transgenes**. The indicated constructs were used to create Shc transgenic mice. ***A***. The human p52 Shc (ShcWT) or ***B***. p52 Shc with mutations of three tyrosine residues (Shc Y239F/Y240F/Y317F; ShcFFF) are fused to a single FLAG tag at the N-terminus. Presence of a STOP cassette prevents expression of the transgene. Cre-mediated recombination at the loxP sites removes the STOP cassette, and the EF-1α promoter drives expression of the transgene.

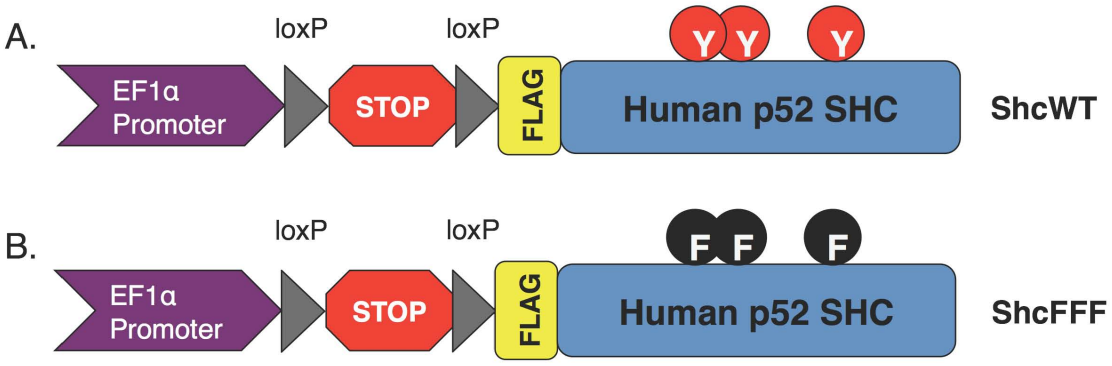

A.
loxP
loxP
Y
Y
Y
EF1α Promoter
STOP
FLAG
Human p52 SHC
ShcWT
B.
loxP
loxP
F
F
F
EF1α Promoter
STOP
FLAG
Human p52 SHC
ShcFFF

Figure 3-2. **Comparison of murine versus human p52 Shc amino acid sequences.** Murine p52 Shc (accession NP_035498) and human p52 Shc (accession NP_003020) were aligned using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). The three critical tyrosine residues of Shc are highlighted in red.

```
Mouse    1   MNKLSGGGGRRTRVEGGQLGGEEWTRHGSFVNKPTRGWLHPNDKVMGPGVSYLVRYMGCV
             MNKLSGGGGRRTRVEGGQLGGEEWTRHGSFVNKPTRGWLHPNDKVMGPGVSYLVRYMGCV
Human    1   MNKLSGGGGRRTRVEGGQLGGEEWTRHGSFVNKPTRGWLHPNDKVMGPGVSYLVRYMGCV

Mouse   61   EVLQSMRALDFNTRTQVTREAISLVCEAVPGAKGATRRRKPCSRPLSSILGRSNLKFAGM
             EVLQSMRALDFNTRTQVTREAISLVCEAVPGAKGATRRRKPCSRPLSSILGRSNLKFAGM
Human   61   EVLQSMRALDFNTRTQVTREAISLVCEAVPGAKGATRRRKPCSRPLSSILGRSNLKFAGM

Mouse  121   PITLTVSTSSLNLMAADCKQIIANHHMQSISFASGGDPDTAEYVAYVAKDPVNQRACHIL
             PITLTVSTSSLNLMAADCKQIIANHHMQSISFASGGDPDTAEYVAYVAKDPVNQRACHIL
Human  121   PITLTVSTSSLNLMAADCKQIIANHHMQSISFASGGDPDTAEYVAYVAKDPVNQRACHIL

Mouse  181   ECPEGLAQDVISTIGQAFELRFKQYLRNPPKLVTPHDRMAGFDGSAWDEEEEEPPDHQYY
             ECPEGLAQDVISTIGQAFELRFKQYLRNPPKLVTPHDRMAGFDGSAWDEEEEEPPDHQYY
Human  181   ECPEGLAQDVISTIGQAFELRFKQYLRNPPKLVTPHDRMAGFDGSAWDEEEEEPPDHQYY

Mouse  241   NDFPGKEPPLGGVVDMRLRE - - - - GAARPTLPSAQMSSHLGATLPIGQHAAGDHEVRKQM
             NDFPGKEPPLGGVVDMRLRE        GAARPT P+AQ    SHLGATLP+GQ     GD EVRKQM
Human  241   NDFPGKEPPLGGVVDMRLREGAAPGAARPTAPNAQTPSHLGATLPVGQPVGGDPEVRKQM

Mouse  297    -LPPPPCPGRELFDDPSYVNIQNLDKARQAGGGAGPPNPSLNGSAPRDLFDMKPFEDALR
                   PPP   GRELFDDPSYVN+QNLDKARQA GGAGPPNP++NGSAPRDLFDMKPFEDALR
Human  301    PPPPPCPAGRELFDDPSYVNVQNLDKARQAVGGAGPPNPAINGSAPRDLFDMKPFEDALR

Mouse  356   VPPPPQSMSMAEQLQGEPWFHGKLSRREAEALLQLNGDFLVRESTTTPGQYVLTGLQSGQ
             VPPPPQS+SMAEQL+GEPWFHGKLSRREAEALLQLNGDFLVRESTTTPGQYVLTGLQSGQ
Human  361   VPPPPQSVSMAEQLRGEPWFHGKLSRREAEALLQLNGDFLVRESTTTPGQYVLTGLQSGQ

Mouse  416   PKHLLLVDPEGVVRTKDHRFESVSHLISYHMDNHLPIISAGSELCLQQPVDRKV
             PKHLLLVDPEGVVRTKDHRFESVSHLISYHMDNHLPIISAGSELCLQQPV+RK+
Human  421   PKHLLLVDPEGVVRTKDHRFESVSHLISYHMDNHLPIISAGSELCLQQPVERKL
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Figure 3-3. **Conditional deletion of murine *Shc1*.** ***A***. Schematic of the murine *Shc1* locus. Exons are indicated as blue boxes; introns are indicated as the line between boxes. Arrowheads indicate *loxP* sites. Exons 1 and 2 of *Shc1* are floxed by *loxP* sites. Cre-mediated recombination removes the intervening DNA sequence. ***B***. Enlarged diagram of the first *loxP* site, indicating disruption of *Cks1* with the *loxP* site. ***C***. Reduced body size of mice homozygous for floxed *Shc1*.

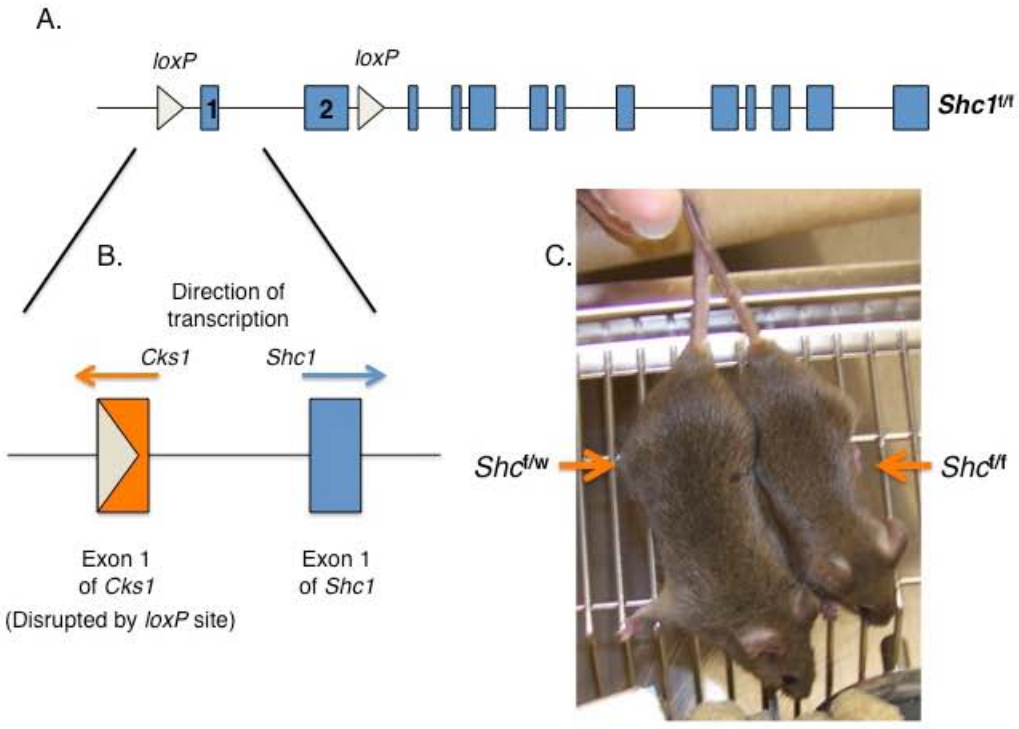

A.
loxP
loxP
1
2
Shc1f/f
B.
Direction of transcription
Cks1
Shc1
Exon 1 of Cks1 (Disrupted by loxP site)
Exon 1 of Shc1
C.
Shcf/w
Shcf/f

Figure 3-4. **Expression of transgenic Cre during B cell development**. B cell development in the adult mouse bone marrow is depicted. Expression of Cre protein for *Mb1*-Cre and *Cd19*-Cre transgenic mice is indicated below the diagram. Dashed line indicates weak expression.

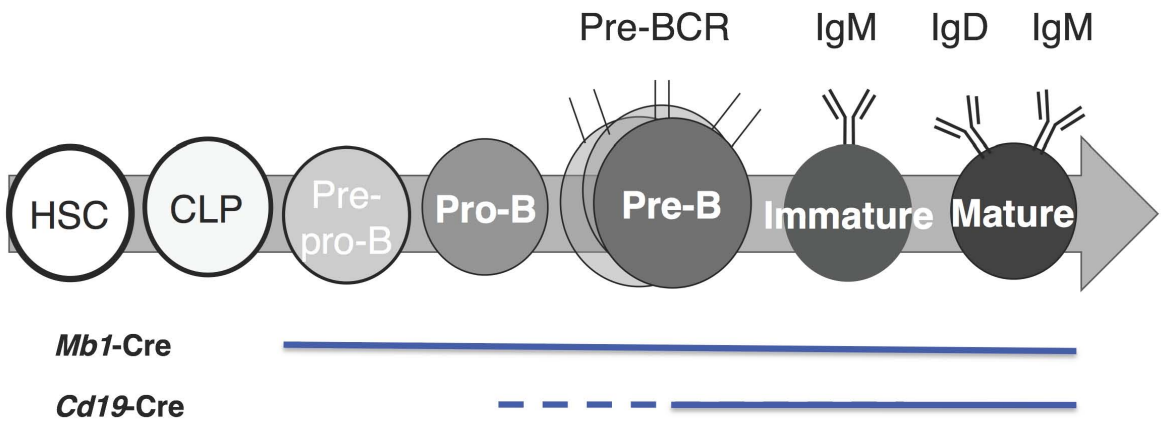
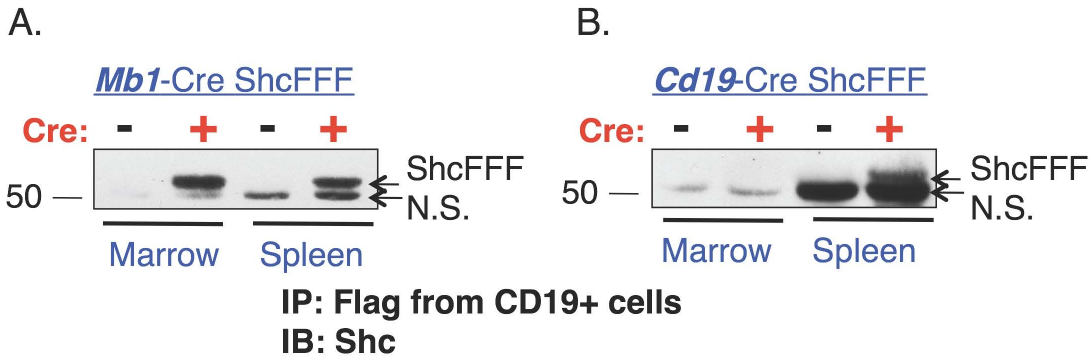

Pre-BCR
IgM
IgD
IgM
HSC
CLP
Pre-pro-B
Pro-B
Pre-B
Immature
Mature
Mb1-Cre
Cd19-Cre

Figure 3-5. **Conditional expression of ShcFFF protein**. $CD19^{+}$ cells were positively selected from bone marrow and spleen of the indicated mice, immunoprecipitated (IP) with anti-FLAG, and immunoblotted (IB) with anti-Shc antibody to detect ShcFFF protein. ***A***. ShcFFF protein is seen in both the bone marrow and spleen of *Mb1*-Cre/ShcFFF mice. ***B***. ShcFFF protein is only observed in the spleen of *Cd19*-Cre/ShcFFF mice. N.S., Nonspecific band.


A.
Mb1-Cre ShcFFF
Cre: - + - +
50 —
ShcFFF
N.S.
Marrow
Spleen
B.
Cd19-Cre ShcFFF
Cre: - + - +
50 —
ShcFFF
N.S.
Marrow
Spleen
IP: Flag from CD19+ cells
IB: Shc

Conditional expression of ShcFFF, but not ShcWT, results in reduced numbers of pro-B cells during B cell development.

My initial examination of *Mb1*-Cre/ShcFFF bone marrow revealed a severe block in B cell development that was first apparent in the pro-B cell compartment (Figure 3-6, A). Importantly, I saw no defect in the HSC compartment, which is prior to *Mb1*-Cre expression. This suggested that the fidelity of Cre, and in turn ShcFFF expression, were retained in *Mb1*-Cre/ShcFFF mice. Additionally, I saw no difference in the numbers of CLPs (Figure 3-6, A). Together, these data suggested that the dominant negative ShcFFF protein was inhibiting B cell development after the CLP but before the pre-B cell stage.

To control for expression of a transgenic Shc protein, I crossed *Mb1*-Cre mice to ShcWT mice, which express a wild-type Shc protein based on the same Cre/*loxP* approach as the ShcFFF mutant (Figure 3-1). *Mb1*-Cre/ShcWT mice displayed no detectable defect in B cell development (Figure 3-6, B).

In contrast to *Mb1*-Cre/ShcFFF mice, conditional expression of ShcFFF under *Cd19*-Cre resulted in no detectable defect in marrow B cell subsets (Figure 3-6, C). Although this finding does not rule out a role for Shc in peripheral B cell function, it suggests that the importance of Shc during B cell development lies in the earlier stages and may not have a similar requirement during later stages of development.

I then sought to narrow the window at which the developmental defect occurred in *Mb1*-Cre/ShcFFF mice. For this, I examined the pre-pro-B compartment, which is an intermediate step between CLP and pro-B stages. Using flow cytometry, I first gated out non-B lineage and IgM^+ B cells ($CD3\varepsilon^-$, $Gr1^-$, $Ter119^-$, $NK1.1^-$, $Ly6C^-$, IgM^-), then gated

on $B220^+$, $CD43^+$, $AA4.1^+$ cells, and finally displayed early B fractions as $CD19^-$ $BP1^-$ (pre-pro-B), $CD19^+$ $BP1^-$ (early pro-B), and $CD19^+$ $BP1^+$ (late pro-B). This revealed a 67% decrease in the early pro-B stage of *Mb1*-Cre/ShcFFF mice compared to littermate controls (Figure 3-7, A). Importantly, this was not seen when the wild-type Shc transgene was expressed in *Mb1*-Cre/ShcWT mice (Figure 3-7, B). Since a consensus in defining the pre-pro-B compartment is lacking, I used multiple gating strategies with flow cytometry but observed no defect in the pre-pro-B compartment of *Mb1*-Cre/ShcFFF mice (Figure 3-8). This places the most proximal defect in B cell development due to impaired Shc-mediated signaling either at the pre-pro-B to early pro-B transition or within cells of the pro-B pool.

Figure 3-6. **ShcFFF expression blocked early B cell development**. Absolute cell numbers were determined from FACS analysis to identify the indicated lymphocyte subset and total bone marrow counts ***A***. *Mb1*-Cre/ShcFFF mice show a developmental block beginning at the pro-B stage (data from five *Mb1*-Cre control and 11 *Mb1*-Cre/ShcFFF mice). ***B***. *Mb1*-Cre/ShcWT mice (eight *Mb1*-Cre and eight *Mb1*-Cre/ShcWT mice) and ***C***. *Cd19*-Cre/ShcFFF mice do not show defects in cellularity of the indicated subsets (data from four *Cd19*-Cre and 10 *Cd19*-Cre/ShcFFF mice). *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$.

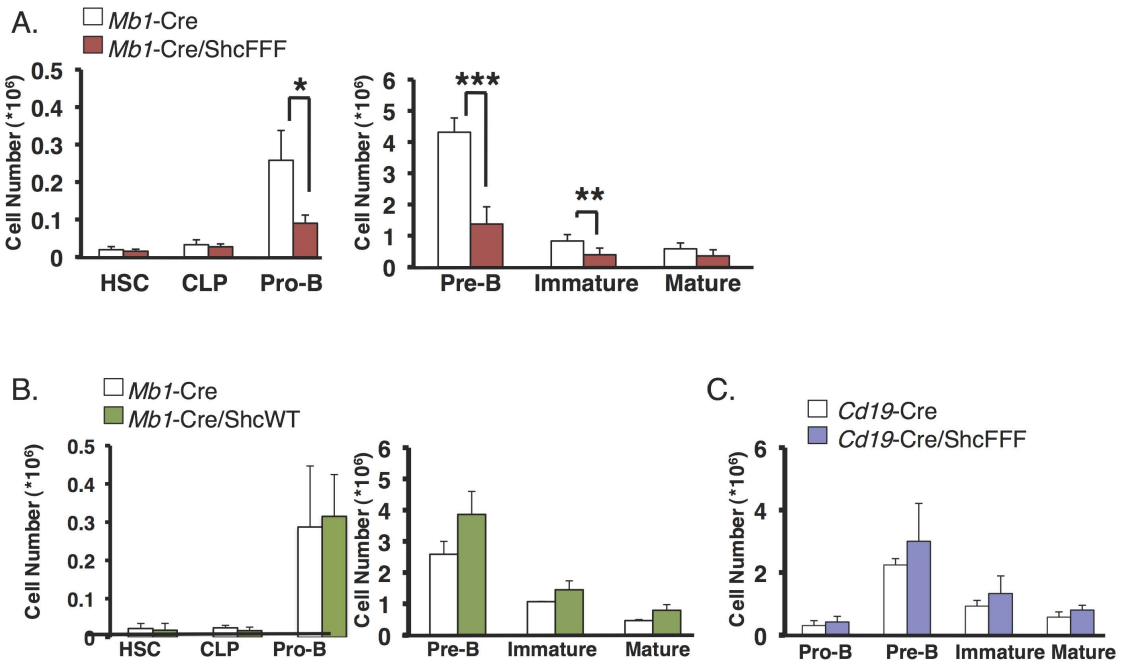

A.
Mb1-Cre
Mb1-Cre/ShcFFF
Cell Number (*10^6)
0.5
0.4
0.3
0.2
0.1
0
*
HSC
CLP
Pro-B
6
5
4
3
2
1
0
***
**
Pre-B
Immature
Mature
B.
Mb1-Cre
Mb1-Cre/ShcWT
C.
Cd19-Cre
Cd19-Cre/ShcFFF
Pro-B
Pre-B
Immature
Mature

Figure 3-7. **Conditional expression of ShcFFF results in reduced numbers of pro-B cells but normal pre-pro-B cell numbers in adult mouse bone marrow**. Absolute cell numbers were determined from FACS analysis to identify the indicated lymphocyte subset and total bone marrow counts ***A***. *Mb1*-Cre/ShcFFF mice show decreased numbers of pro-B cells (data from seven *Mb1*-Cre and six *Mb1*-Cre/ShcFFF mice). ***B***. *Mb1*-Cre/ShcWT mice do not show defects in cellularity of the indicated subsets (data from six *Mb1*-Cre and four *Mb1*-Cre/ShcWT mice). **, $p < 0.001$.

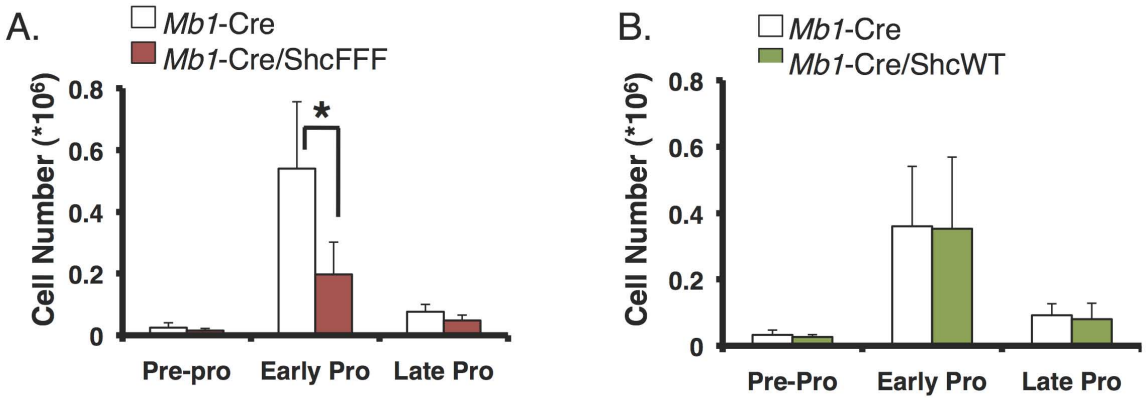
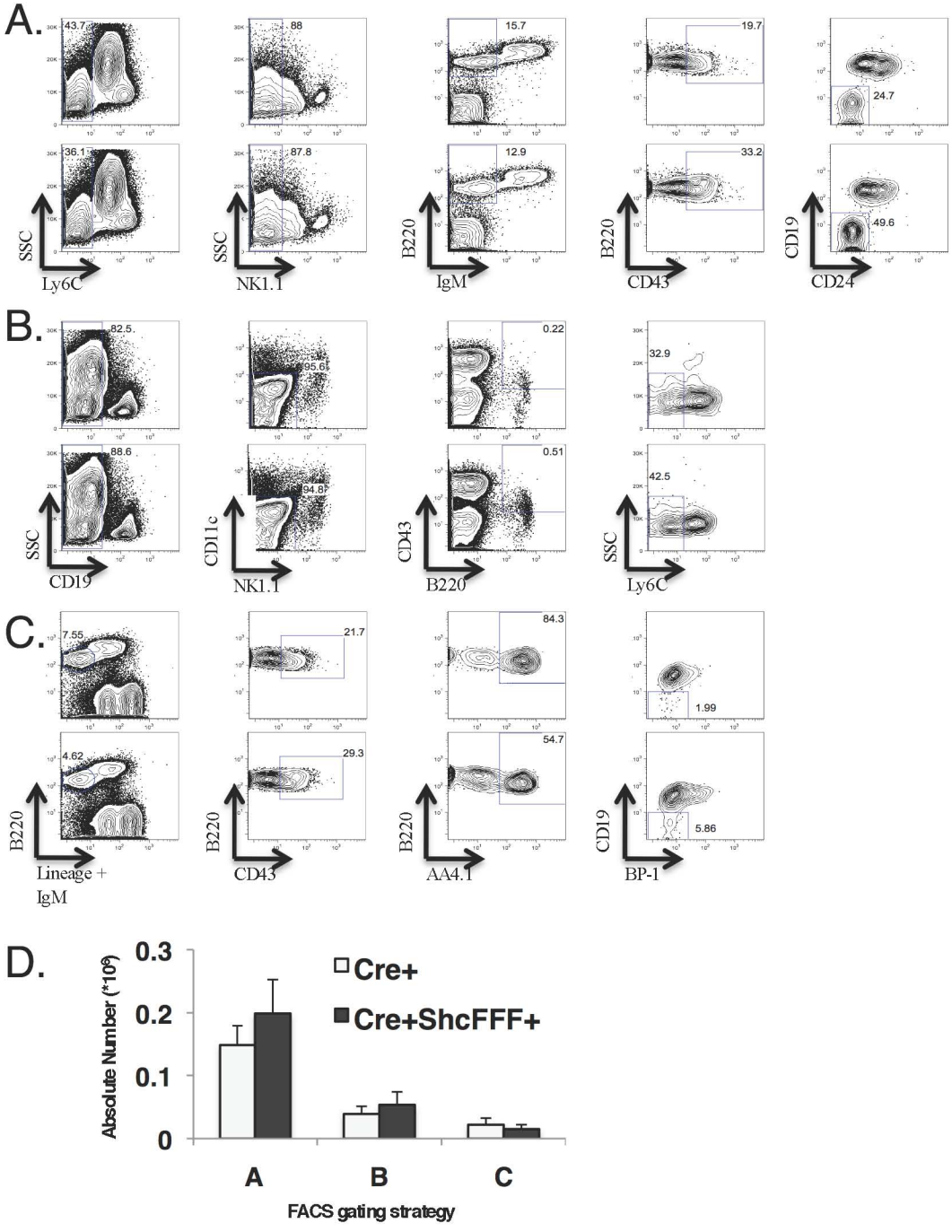

A.
Mb1-Cre
Mb1-Cre/ShcFFF
Cell Number (*10^6)
0.8
0.6
0.4
0.2
0
*
Pre-pro
Early Pro
Late Pro
B.
Mb1-Cre
Mb1-Cre/ShcWT
Cell Number (*10^6)
0.8
0.6
0.4
0.2
0
Pre-Pro
Early Pro
Late Pro

Figure 3-8. **Normal pre-pro-B numbers in *Mb1*-Cre/ShcFFF mice using multiple FACS gating strategies**. Absolute cell numbers were determined from FACS analysis to determine total cellularity of pre-pro-B compartment ***A-C***. Representative FACS gating strategies used to identify pre-pro-B subsets. Previous gating included singlets (based on pulse width versus FSC) and live cells (based on SSC versus DAPI exclusion). ***D***. Total cell numbers determined from the indicated FACS gating analysis. A total of seven *Mb1*-Cre and five *Mb1*-Cre/ShcFFF mice were used for this experiment.


A.
43.7
88
15.7
19.7
24.7
36.1
87.8
12.9
33.2
49.6
SSC
Ly6C
SSC
NK1.1
B220
IgM
B220
CD43
CD19
CD24
B.
82.5
0.22
32.9
88.6
95.6
94.8
0.51
42.5
SSC
CD19
CD11c
NK1.1
CD43
B220
SSC
Ly6C
C.
7.55
21.7
84.3
1.99
4.62
29.3
54.7
5.86
B220
Lineage +
IgM
B220
CD43
B220
AA4.1
CD19
BP-1
D.
Absolute Number (*10^6)
0.3
0.2
0.1
0
Cre+
Cre+ShcFFF+
A
B
C
FACS gating strategy

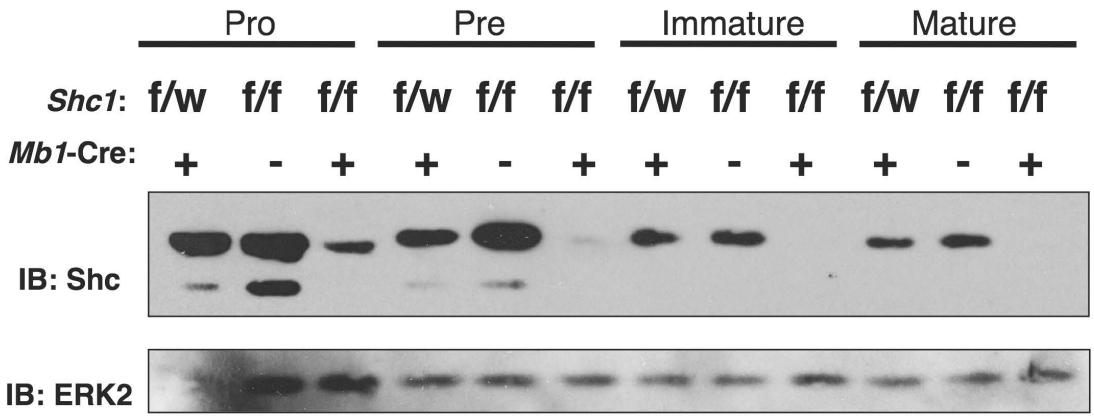
Conditional deletion of* Shc1 *also blocks early B cell development

In addition to conditional expression of transgenic Shc proteins, I also examined the requirement for Shc during B cell development with conditional *Shc1* knockout mice ($Shc^{f/f}$) (Figure 3-3). Although Shc protein was efficiently removed in sorted bone marrow B cells beginning at the pre-B stage, I noticed residual Shc protein in the pro-B subset of *Mb1*-Cre/$Shc^{f/f}$ mice (Figure 3-9). In addition, there was not a reproducible, statistically significant defect in B cell subsets in the bone marrow or spleen of these mice (Figure 3-10).

Senescence can allow subtle defects in B cell development to become more apparent, as CLPs, pre-pro-B, and pro-B cell numbers and proliferative capacity are reduced in aged mice [199]. Since residual Shc protein was present in pro-B cells of *Mb1*-Cre/$Shc^{f/f}$ mice, I examined mice that were allowed to age to 9 months. Aged *Mb1*-Cre/$Shc^{f/f}$ mice showed developmental defects in the bone marrow beginning at the pro-B stage (Figure 3-10), consistent with *Mb1*-Cre/ShcFFF mice. This demonstrates that both Shc protein and its ability to be tyrosine phosphorylated on the three critical tyrosine residues are required for early B cell development.

Figure 3-9. **Conditional deletion of *Shc1* and subsequent loss of Shc protein**. Bone marrow from littermate mice from the indicated genotypes were sorted based on FACS gating established in earlier figures. Total lysates were analyzed by Western blotting for total Shc protein. Erk2 was used as a loading control. This is representative of two experiments.

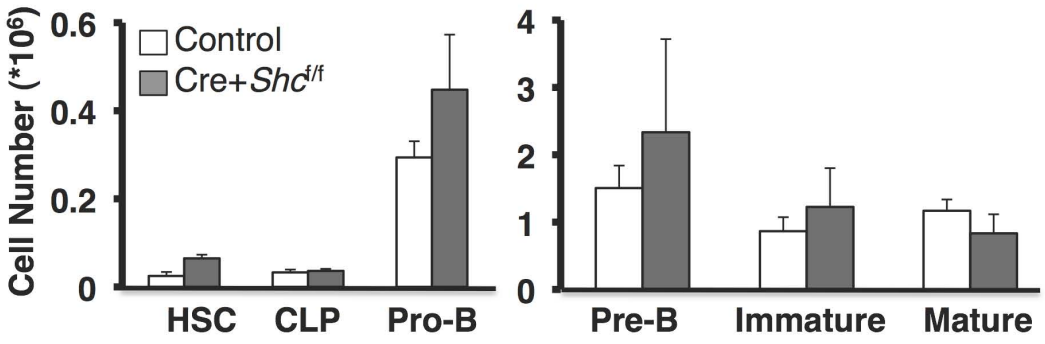
Conditional deletion of *Shc1* and subsequent loss of Shc protein



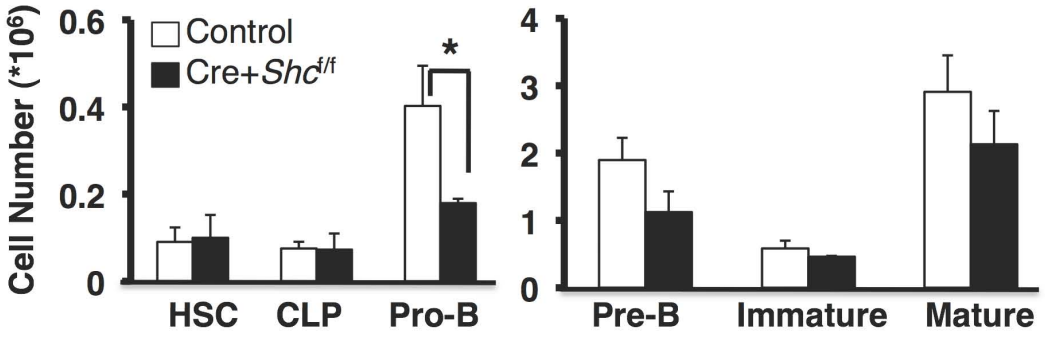
Total lysates of FACS sorted marrow from littermate mice

Figure 3-10. **Conditional deletion of *Shc1* blocks early B cell development in aged mice.** Absolute cell numbers were determined from FACS analysis to identify the indicated lymphocyte subset and total bone marrow counts ***A***. Eight-week-old *Mb1*-Cre/*Shc*$^{f/f}$ mice do not show a developmental block in the bone marrow (data from two *Shc*$^{f/f}$ control and six *Mb1*-Cre/*Shc*$^{f/f}$ mice). ***B***. Aged *Mb1*-Cre/*Shc*$^{f/f}$ mice show diminished pro-B cell numbers (data from two *Shc*$^{f/w}$ control and two *Mb1*-Cre/*Shc*$^{f/f}$ mice). *, $p < 0.01$.

A. ***Mb1*-Cre *Shc* flox (8 weeks)**



B. ***Mb1*-Cre *Shc* flox (9months)**



Diminished B cell compartment in the spleen of* Mb1-*Cre/ShcFFF mice

Immature B cells from the bone marrow populate the spleen as transitional B cells. Transitional B cells are nonproliferative, functionally immature B cells and undergo measurable cell loss as they mature [42, 44, 200]. Analysis of splenic B cell subsets from *Mb1*-Cre/ShcFFF mice revealed decreased immature, $AA4.1^{+}$ transitional B cells (T1, T2, and T3 based on IgM versus CD23 expression) and $AA4.1^{-}$ mature follicular B cells (though not statistically significant), but no apparent defect in marginal zone B cells or T cells (Figure 3-11, A).

Importantly, B cell development was not impaired in mice conditionally expressing the wild-type Shc transgene (Figure 3-11, B). Moreover, when I assessed the spleen of mice expressing ShcFFF under *Cd19*-Cre, I saw no defect in splenic B cell subsets (Figure 3-11, C). Finally, the spleen of 8-week-old *Mb1*-Cre/$Shc^{f/f}$ mice did not show an apparent defect in B cell subsets although efficient deletion of Shc protein was observed by the pre-B cell stage (Figure 3-12, A). However, when I allowed the mice to age to 9 months I noticed diminished numbers of B cells in the spleen (Figure 3-12, B). Taken together, these studies point to a critical role for Shc-mediated signaling predominantly during early B cell development rather than later maturational events.

Figure 3-11. **Block in *Mb1*-Cre/ShcFFF B cells is maintained in the spleen**. Absolute cell numbers were determined from FACS analysis to identify the indicated lymphocyte subset and total splenocyte counts ***A***. *Mb1*-Cre/ShcFFF mice have diminished transitional (T1-T3) B cells but normal marginal zone (MZ) B cells and T cells (data from five *Mb1*-Cre control and 11 *Mb1*-Cre/ShcFFF mice). ***B***. *Mb1*-Cre/ShcWT mice (data from eight *Mb1*-Cre and eight *Mb1*-Cre/ShcWT mice) and ***C***. *Cd19*-Cre/ShcFFF mice (data from four *Cd19*-Cre and 10 *Cd19*-Cre/ShcFFF mice) do not show defects in cellularity of the indicated subsets. *, $p < 0.01$; **, $p < 0.001$.

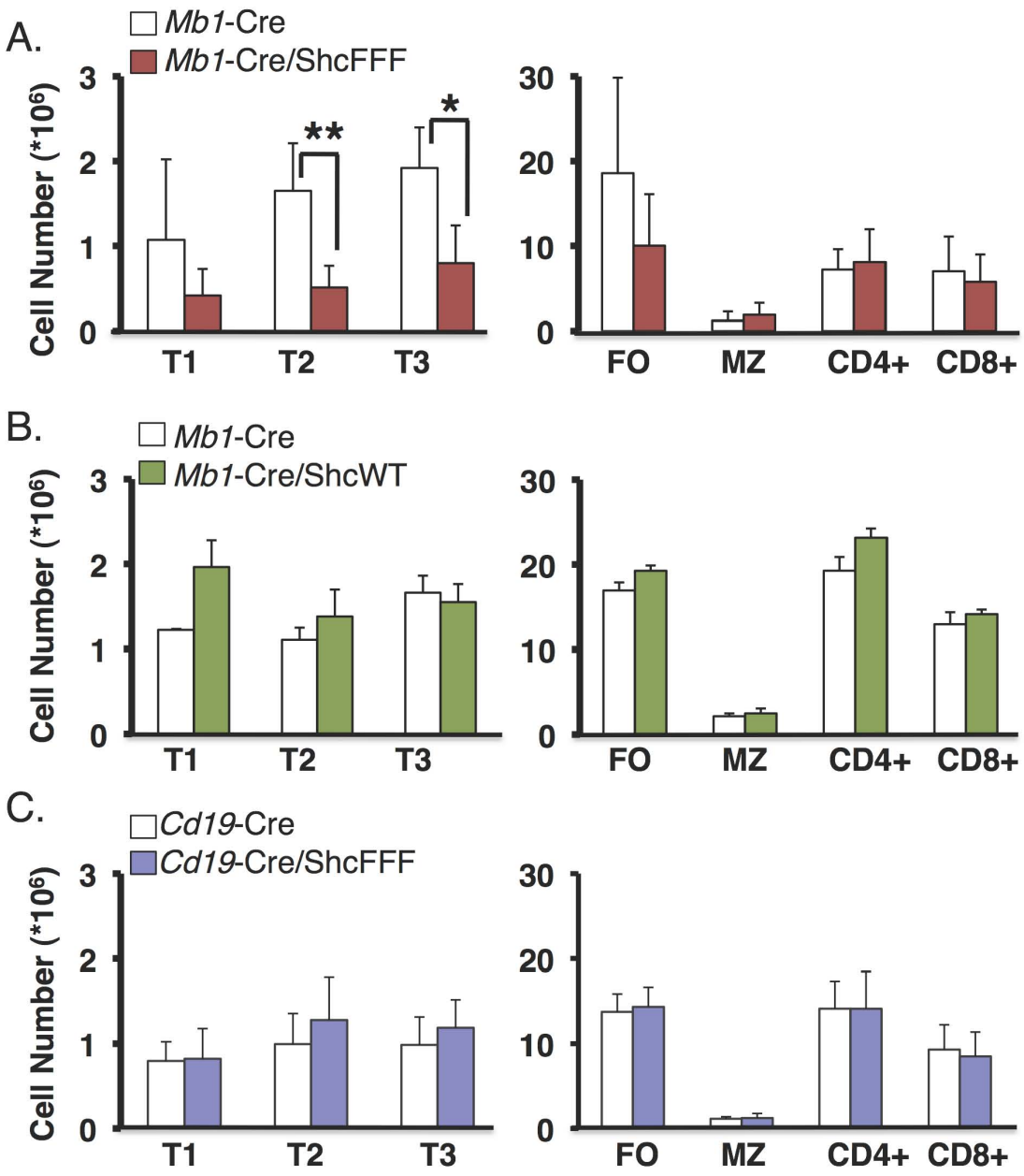
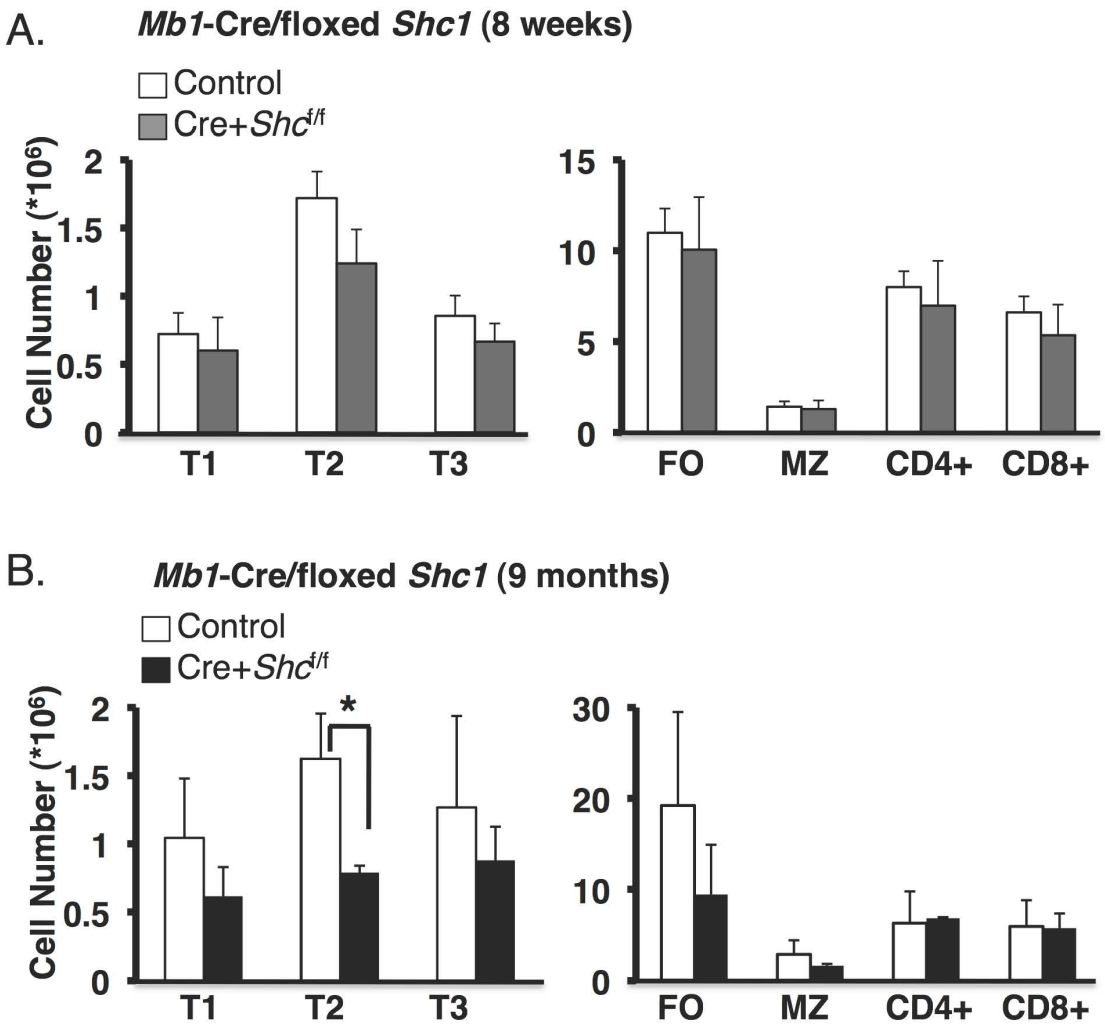

A.
Mb1-Cre
Mb1-Cre/ShcFFF
Cell Number (*10^6)
3
2
1
0
**
*
T1
T2
T3
30
20
10
0
FO
MZ
CD4+
CD8+
B.
Mb1-Cre
Mb1-Cre/ShcWT
Cell Number (*10^6)
3
2
1
0
T1
T2
T3
30
20
10
0
FO
MZ
CD4+
CD8+
C.
Cd19-Cre
Cd19-Cre/ShcFFF
Cell Number (*10^6)
3
2
1
0
T1
T2
T3
30
20
10
0
FO
MZ
CD4+
CD8+

Figure 3-12. **Loss of Shc is correlated with diminished splenic B numbers in aged mice**. Absolute cell numbers were determined from FACS analysis to identify the indicated lymphocyte subset and total splenocyte counts ***A***. Eight-week-old *Mb1*-Cre/*Shc*$^{f/f}$ mice have splenic B numbers relative to littermates (data from two *Shc*$^{f/f}$ control and six *Mb1*-Cre/*Shc*$^{f/f}$ mice). ***B***. Aged *Mb1*-Cre/*Shc*$^{f/f}$ mice have decreased transitional (T1-T3) and follicular (FO) B cells but normal marginal zone (MZ) and T cells, similar to *Mb1*-Cre/ShcFFF mice (data from two control and two *Mb1*-Cre/*Shc*$^{f/f}$ mice). *, $p < 0.01$.


A.
Mb1-Cre/floxed Shc1 (8 weeks)
Control
Cre+Shcf/f
Cell Number (*10^6)
2
1.5
1
0.5
0
T1
T2
T3
15
10
5
0
FO
MZ
CD4+
CD8+
B.
Mb1-Cre/floxed Shc1 (9 months)
Control
Cre+Shcf/f
Cell Number (*10^6)
*
30
20
10
0

Discussion

The adaptor protein Shc was initially shown to become phosphorylated in B cell lines in response to BCR and BCR + FcR ligation [195, 201-203]. However, due to conflicting results seen in B cell lines [196] and the lack of studies in less mature B cells, the role of Shc in primary B cell development has remained unresolved. By utilizing the Cre/*loxP* system, I discovered a novel requirement for Shc during early B cell development.

Based upon previous work in our lab that identified Shc as playing a key role during the β selection checkpoint[174], I had originally hypothesized that the first requirement for Shc would be at the homologous pro-B to pre-B transition during the pre-BCR checkpoint. However, disruption of Shc by conditional deletion of *Shc1* or transgenic expression of ShcFFF led to a significant reduction in pro-B cell numbers. This is earlier than the anticipated pro-B to pre-B transition, in which a functional pre-BCR is required for developmental progression. Although critical roles for cell surface receptors, such as cytokine receptors, have been demonstrated during this stage of development, the requirements for intracellular components responsible for the pleiotropic effects of these receptors remains incomplete. Previous studies have demonstrated the requirements of other adaptor proteins during multiple stages of development, yet their functional significance is found to lie beyond the pro-B stage [124-126, 128-130]. This made the phenotype observed in mice with disruption of Shc during early B cell development particularly interesting.

B cell development in *Cd19*-Cre/ShcFFF mice and eight-week-old *Mb1*-Cre/*Shc1*$^{f/f}$ mice appeared normal. Each of these mice has caveats in the bone

marrow. *Cd19*-Cre/ShcFFF mice have undetectable levels of ShcFFF protein, although this does not rule out the presence of ShcFFF. ShcFFF protein may only be present in low levels, and/or cells expressing ShcFFF may be eliminated. However, ShcFFF protein is clearly present in splenic B cells. *Mb1*-Cre/*Shc1*$^{f/f}$ mice have detectable endogenous Shc protein in the pro-B cell subset, but it is absent by the pre-B cell stage. Each of these mouse models potentially maintains normal Shc signaling during early B cell development but disrupted Shc signaling by the pre-B cell stage or later. Likewise, each of these mouse models shows normal splenic B cell numbers. This suggests that Shc and Shc phosphorylation may play a redundant role in B cell development beyond the pre-B stage. However, this does not rule out a requirement for Shc during peripheral B cell function. Since Shc is phosphorylated in response to both positive (BCR) and negative (BCR+FcR) signals, it remains a potentially important signaling intermediate during the B cell response to positive and negative stimulation.

Chapter IV

Disruption of Shc signaling causes abnormal apoptosis of early B subsets

I had discovered that disruption of Shc signaling blocked B cell development at the pre-pro-B to pro-B transition, which is prior to the pre-BCR selection checkpoint (Chapter III). To understand how ShcFFF disrupted B cell development, I once again based my hypothesis on previous work in which ShcFFF blocked T cell development by disrupting the proliferative burst that accompanies successful pre-TCR rearrangement. However, I was surprised to observe that proliferation appeared normal in ShcFFF-expressing B cells as demonstrated by multiple assays. Instead, I found a defect in survival of ShcFFF-expressing B cells. These findings were also published in [175].

Abstract

Disruption of Shc signaling during B cell development resulted in decreased cell numbers of early B subsets. These depressed B cell numbers could have resulted from defects in proliferation, differentiation, survival, or commitment of these early cells to the B lineage. Since Shc is involved in signal transduction from multiple cell surface receptors, I chose to test receptor signaling further. The initial block in mice with disrupted Shc signaling occurred prior to the pre-BCR checkpoint, so I focused on cytokine receptors, which mediate multiple responses in early B development. In particular, the cytokine IL-7 has been extensively studied for its importance during early

B cell development. In this chapter, I found the ShcFFF-expressing B cells are deficient in their ability to respond to IL-7. These B cells abnormally undergo apoptosis when cultured in IL-7-containing medium. Furthermore, this defect is independent of the pre-BCR. Therefore, unlike T cells, which show impaired proliferation due to disrupted Shc-mediated signaling via the pre-TCR, early B cells undergo apoptosis due to an inability to respond to IL-7.

Introduction

In the previous chapter, I determined that disruption of Shc had led to a developmental block in murine bone marrow. This was manifested in reduced numbers of B subsets in the bone marrow beginning with the pro-B stage. Compared to the requirements for B cell development at the pre-BCR checkpoint and beyond, relatively little has been published on B cell development prior to expression of the pre-BCR. I could not rule out a requirement for Shc in signaling through the pre-BCR, but the initial developmental defect observed within the pro-B subset was highly unlikely to be a result of impaired pre-BCR signaling.

Signaling through the IL-7 receptor is critical for B cell development beyond the pre-pro-B cell stage. The IL-7 receptor has been reported to initiate signaling through the PI-3K [89, 204, 205], ERK/MAPK [90, 206], STAT1 [206], and STAT5 [207] signaling pathways. IL-7 receptor stimulation activates the Janus kinases Jak1 and Jak3 [206, 208]. Activation of Jak kinases in response to IL-7 stimulation can lead to phosphorylation of Stat1 and Stat5 [209]. Following activation, Stats dimerize and bind DNA to regulate gene transcription (reviewed in [210]). The *Pax5* promoter region contains a Stat-binding motif [211]. In addition, *Stat5a/Stat5b* double knockout mice fetal liver contains reduced expression of *Ebf* and *Pax5*, and B cell development is arrested at the pre-pro-B cell stage [212, 213]. These findings link activation of Stats to B lineage commitment during B cell development. Transgenic expression of a constitutively active Stat5b construct results in increased pro-B cell numbers. Additionally, expression of the constitutively active Stat5b on an *IL-7R*$^{-/-}$ background increased pro-B cell numbers nearly six fold over an *IL-7R*$^{-/-}$ mouse. However, even with

the presence of the constitutively active Stat5 construct, pro-B cell numbers in mice on an *IL-7R*$^{-/-}$ background were still only 34% of the number of littermate controls [207]. Therefore, Stat5 clearly plays a role in signaling downstream from the IL-7 receptor, but it cannot compensate for all the effects of IL-7 signaling.

Mice deficient in the p85α regulatory subunit of PI-3K exhibit impaired B cell development in the bone marrow near the pro-B cell stage [172], although detailed FACS analyses are lacking. Functional responses of mature B cells from *p85α*$^{-/-}$ mice to LPS, anti-IgM, and anti-CD40 ligation are impaired. The *p85α* gene encodes three splice variants in mice: p85α, p55α, and p50α. Mice with disruption of all three regulatory isoforms also display disruption during early B cell development [173]. Mature B cells from *p85α*$^{-/-}$*p55α*$^{-/-}$*p50α*$^{-/-}$ mice or littermate controls treated with the PI-3K inhibitor Ly294002 incorporate [^{3}H]-thymidine to a much lesser extent than littermate controls in response to multiple stimuli, including anti-IgM crosslinking and LPS. In addition to decreased [^{3}H]-thymidine incorporation, mature B cells have an increased percentage of cells within the sub-2n gate (a FACS gating strategy to identify cells undergoing DNA degradation during death) when cultured with or without mitogenic stimuli [173]. Together, these data suggest a potential role for PI3K during early B cell development, although this protein appears to be largely important during the proliferative response of more mature B cells.

Expression of a dominant negative Ras transgene (*H-RASN17*) has been shown to block B cell development at the pre-pro-B to pro-B transition and also results in reduced numbers of pre-pro-B cells [120, 121]. This block is mildly alleviated by transgenic expression of constitutively active Raf (*Raf-CAAX*). These data suggest a critical role for

Ras during early B cell development, with particular importance at the pre-pro-B to pro-B transition. However, the downstream signaling pathways that were disrupted in these dominant negative Ras mouse models have not been isolated.

In this chapter I sought to gain insight into the mechanism of the developmental block in mice with conditional disruption of Shc signaling. I used a combination of *in vivo* and *ex vivo* approaches to tease apart potential causes for decreased cellularity. Reduced cell numbers could result from impaired proliferation, differentiation, survival, or commitment to the B lineage. Signaling through the IL-7 receptor is required for transition from the pre-pro-B to pro-B transition. In addition, IL-7 can promote proliferation, differentiation, survival, and commitment of early B progenitors to the B lineage. Pro-B cells from *Mb1*-Cre/ShcFFF mice had an impaired response to *ex vivo* culture with IL-7. This disrupted response was independent of the pre-BCR. I determined that ShcFFF-expressing pro-B cells abnormally underwent apoptosis when cultured with IL-7. In addition, pro-B cells within the normal bone marrow milieu retained normal turnover rates but showed decreased production of early lymphocyte subsets. Furthermore, these early lymphocyte subsets displayed increased Annexin V staining. Therefore, ShcFFF-expressing pro-B cells in the normal bone marrow microenvironment as well as those cultured with IL-7 displayed increased apoptosis yet retained a normal turnover rate. Shc is phosphorylated in *Rag1*$^{-/-}$ pro-B cells in response to IL-7 stimulation. These data suggest that phosphorylation of Shc plays a previously unknown role during IL-7 stimulation leading specifically to survival. Other effects of IL-7 stimulation, such as proliferation and commitment to the B lineage remain in tact.

Disruption of IL-7 signaling may result in the developmental defect observed in mice with conditional disruption of Shc signaling.

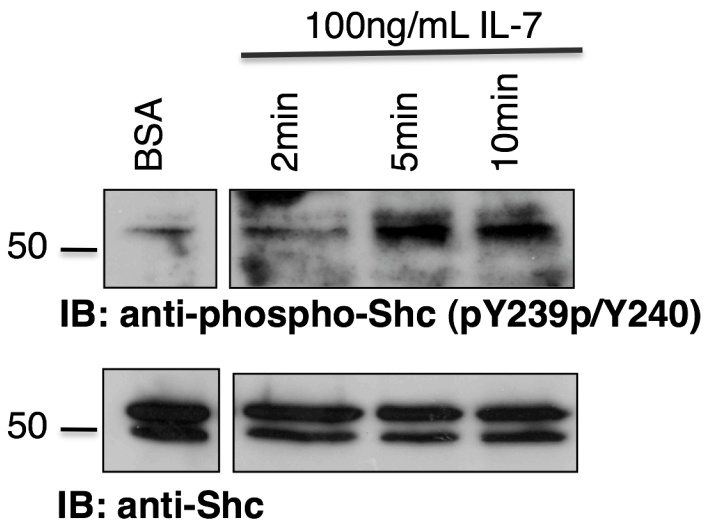
Results

Shc is phosphorylated in response to IL-7 stimulation

To determine whether Shc was phosphorylated in response to IL-7 stimulation, I utilized bone marrow from *Rag1*$^{-/-}$ mice. I chose these mice for this experiment for two specific reasons. First, B cells in *Rag1*$^{-/-}$ mice cannot differentiate beyond the pro-B stage. Pro-B cells are the first early B lymphocytes to express CD19 on the cell surface. Therefore, when I used magnetic selection to isolate bone marrow cells conjugated to anti-CD19 microbeads, I obtained only the pro-B population. Second, it has been documented that the pre-BCR can positively influence the response of cells to IL-7 stimulation [59]. Since I had not ruled out a role for Shc in signaling through the pre-BCR, I wanted to eliminate any influences of this receptor on Shc phosphorylation in response to IL-7 stimulation.

Pro-B cells freshly isolated from *Rag1*$^{-/-}$ bone marrow were stimulated with 100ng/mL of recombinant IL-7 for varying times, and total lysates were analyzed by Western blotting for the presence of phosphorylated Shc (Figure 4-1). I was able to observe a reproducible induction of Shc phosphorylation over time, beginning at 2 minutes, maximal at 5 minutes, and still strongly observed at 10 minutes. The kinetics of Shc phosphorylation are slightly delayed compared with Shc phosphorylation in response to engagement of other receptors (i.e. anti-CD3 stimulation of T cells results in Shc phosphorylation near maximum by 2 minutes post-stimulation, personal observation), yet a clear increase in Shc phosphorylation is observed in response to IL-7 stimulation in pro-B cells. This suggested that pro-B cells could require Shc phosphorylation to respond to IL-7 stimulation.

Figure 4-1. **Shc is tyrosine phosphorylated in response to IL-7 stimulation**. $CD19^{+}$ cells from *Rag1*$^{-/-}$ bone marrow were allowed to rest 1 hr in plain RPMI in a 37°C incubator before stimulation with either BSA or BSA with 100ng/mL IL-7 in a 37°C water bath. Cells were stimulated with IL-7 for the indicated times. Cells receiving BSA (as an unstimulated sample) were incubated for 5 minutes in the 37°C water bath.


100ng/mL IL-7
BSA
2min
5min
10min
50
IB: anti-phospho-Shc (pY239p/Y240)
50
IB: anti-Shc

Mb1-Cre/ShcFFF mice have normal IL-7 receptor expression

As discussed in the introduction, disruption of IL-7 or its receptor leads to a block in B cell development at the pre-pro-B to pro-B transition. I first assessed the expression of the IL-7Rα (CD127) on normal murine early lymphocyte subsets. FACS analysis of normal mouse bone marrow showed that the IL-7Rα was expressed at low levels on pre-pro-B cells and dramatically increased in pro-B cells. By the pre-B cell stage, IL-7Rα expression had begun to decrease again, perhaps demonstrating a switch from IL-7 dependency to pre-BCR selection (Figure 4-2A). The expression of the IL-7Rα chain nicely correlated with the requirement for IL-7 during B cell development.

Before assessing if B cells from *Mb1*-Cre/ShcFFF mice could responds to IL-7, I checked for expression of the IL-7Rα chain on early B subsets in ShcFFF-expressing mice (Figure 4-2, B). I examined developmental subsets from the pre-pro-B to pre-B stages and saw the *Mb1*-Cre/ShcFFF mice expressed levels of the IL-7Rα chain that were comparable to *Mb1*-Cre littermate controls. Importantly, the IL-7Rα was upregulated at the pro-B cell stage.

Mb1-Cre/ShcFFF mice have an impaired response to IL-7 stimulation

To determine if *Mb1*-Cre/ShcFFF pro-B cells could respond to IL-7 stimulation, I isolated pro-B cells from *Mb1*-Cre/ShcFFF mice and littermate controls by FACS-based cell sorting. These cells were plated in varying concentrations of IL-7 and assessed for proliferation by [^{3}H]-thymidine incorporation after four days of culture. Growth medium containing the original concentration of IL-7 were replaced on the third day to refresh IL-7. *Mb1*-Cre/ShcFFF pro-B cells showed decreased [^{3}H]-thymidine uptake compared

to littermate controls across multiple concentrations of IL-7 (Figure 4-3), suggesting a potential explanation for the developmental block in *Mb1*-Cre/ShcFFF bone marrow.

While this experiment was extremely encouraging, it had two major caveats. First, although I had initially plated pro-B cells, these cells are capable of differentiating in IL-7-containing media, resulting in a mixed population of cells over time. Since more mature B cell subsets are less responsive to IL-7, this could have influenced the assay. Second, expression of the pre-BCR can positively influence the response of cells to low concentrations of IL-7. As demonstrated in Figure 4-4, *Rag1*$^{-/-}$ pro-B cells are sensitive to low concentrations of IL-7, as they lack a pre-BCR. Furthermore, they are developmentally arrested at the pro-B cell stage, and higher concentrations of IL-7 allow expansion of pro-B cells. To avoid these caveats, I crossed *Mb1*-Cre/ShcFFF mice onto a *Rag1*$^{-/-}$ background.

Figure 4-2. **ShcFFF does not disrupt surface expression of IL-7Rα**. ***A***. Normal expression of the IL-7Rα chain on early B lymphocytes. $B220^-$ cells are used as a negative control for IL-7Rα staining. ***B***. Comparable expression of IL-7Rα on *Mb1*-Cre/ShcFFF bone marrow B cell subsets compared to littermate control.

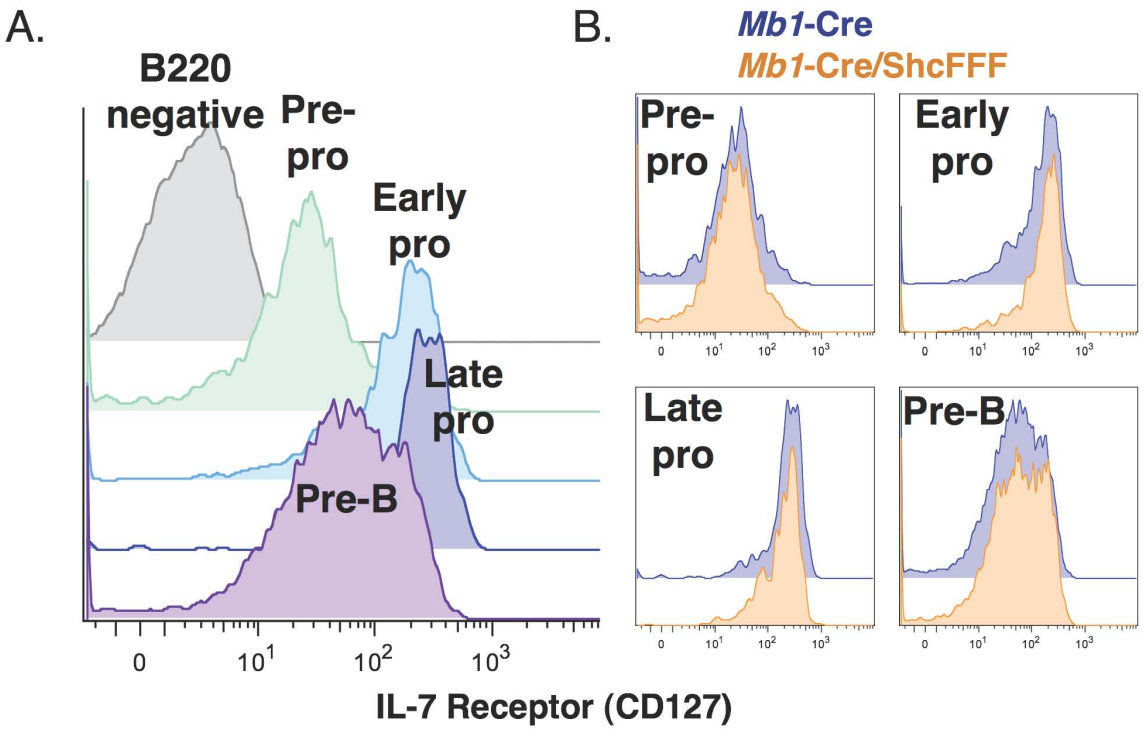

A.
B220 negative
Pre-pro
Early pro
Late pro
Pre-B
0
10^1
10^2
10^3
IL-7 Receptor (CD127)
B.
Mb1-Cre
Mb1-Cre/ShcFFF
Pre-pro
Early pro
Late pro
Pre-B

Figure 4-3. ***Mb1*-Cre/ShcFFF pro-B cells have impaired response to culture with IL-7**. FACS-sorted pro-B cells from littermate mice with the indicated genotype were cultured with varying concentrations of IL-7. On the fourth day of culture, cells were pulsed for 8 hours with 1 μCi of [^{3}H]-thymidine, and [^{3}H]-thymidine incorporation was determined. *, $p < 0.01$; **, $p < 0.001$. This is representative of three independent experiments.

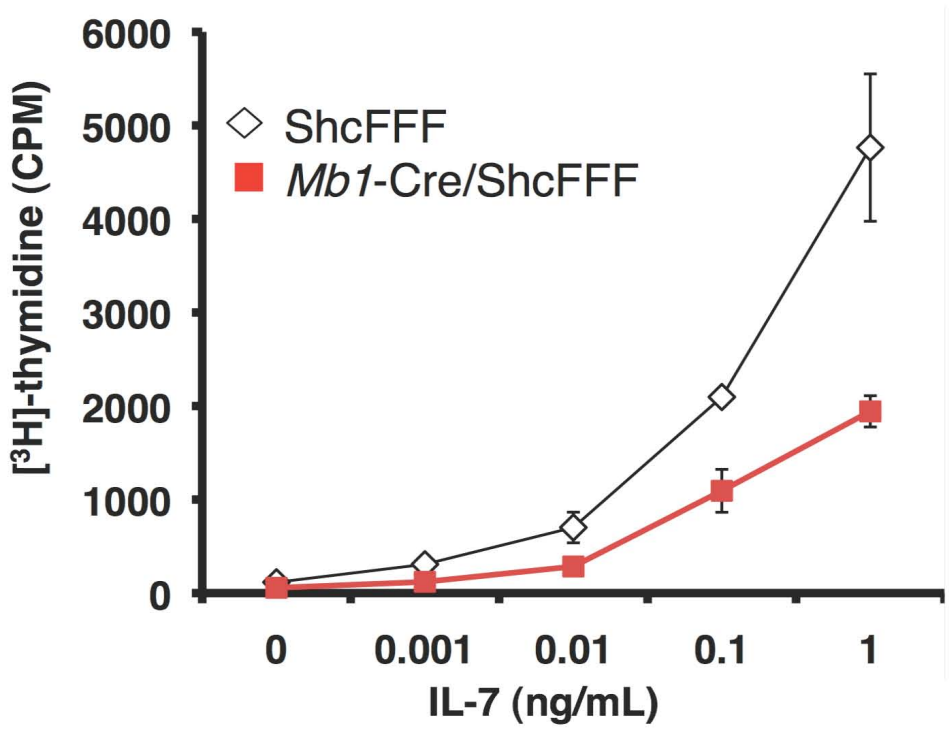
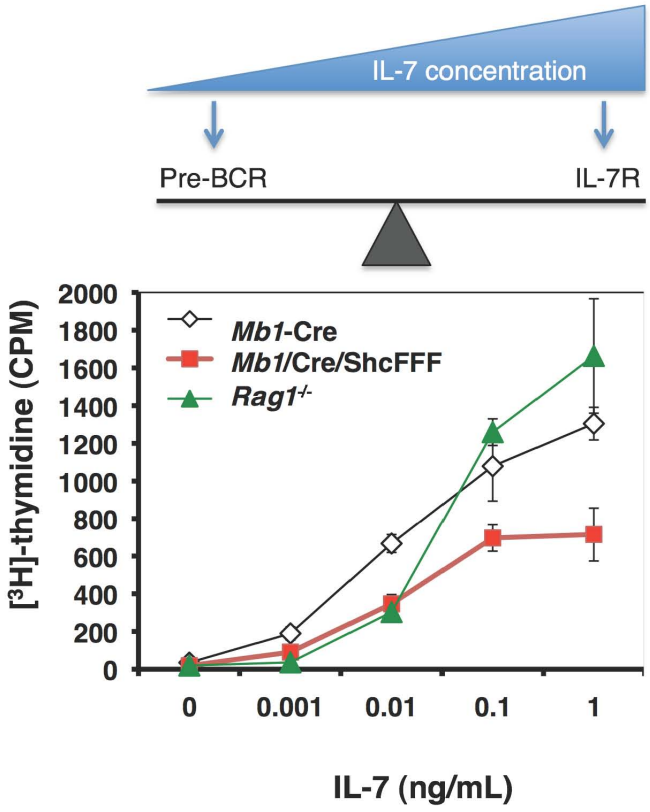

6000
5000
4000
3000
2000
1000
0
[³H]-thymidine (CPM)
ShcFFF
Mb1-Cre/ShcFFF
0
0.001
0.01
0.1
1
IL-7 (ng/mL)

Figure 4-4. ***Mb1*-Cre/ShcFFF pro-B cells have impaired response at low and high concentrations of IL-7.** FACS-sorted pro-B cells from littermate mice with the indicated genotype were cultured with varying concentrations of IL-7. $CD19^{+}$ pro-B cells from $Rag1^{-/-}$ bone marrow were used as a pre-BCR^{-} constant pro-B population control. On the fourth day of culture, cells were pulsed for 8 hours with 1 μCi of [^{3}H]-thymidine, and [^{3}H]-thymidine incorporation was determined.



At low concentrations of IL-7, cells expressing the pre-BCR have a selective advantage. At higher concentrations of IL-7, the advantage is given to cells with a functional IL-7 receptor.

Impaired response to IL-7 is independent of the pre-BCR in Mb1-Cre/ShcFFF/Rag1$^{-/-}$ mice

Mb1-Cre/ShcFFF/*Rag1*$^{-/-}$ mice were analyzed for B cell development in the bone marrow. These mice displayed decreased cellularity in the pro-B compartment (Figure 4-5). This block is at the same stage as observed in *Mb1*-Cre/ShcFFF mice, supporting that decreased pro-B cell number was independent of the pre-BCR. In addition, pro-B cells from these mice also expressed surface levels of the IL-7Rα at levels comparable to littermate controls (Figure 4-6).

I tested the ability of pro-B cells from *Mb1*-Cre/ShcFFF/*Rag1*$^{-/-}$ mice to respond to varying concentrations of IL-7. As observed with *Mb1*-Cre/ShcFFF pro-B cells, the pro-B cells from *Mb1*-Cre/ShcFFF/*Rag1*$^{-/-}$ mice incorporated less [^{3}H]-thymidine compared to littermate controls over a wide range of concentrations (Figure 4-7, A). Interestingly, the defect was even more pronounced in mice lacking V(D)J recombinase machinery. This suggests that pre-BCR may have provided a positive influence on pro-B cells from *Mb1*-Cre/ShcFFF mice, although the precise reasons cannot be determined from this experiment.

In addition to diminished [^{3}H]-thymidine uptake, pro-B cells from *Mb1*-Cre/ShcFFF/*Rag1*$^{-/-}$ mice failed to accumulate in number compared to littermate controls (Figure 4-7, B). This suggests that expression of ShcFFF impairs the ability of pro-B cells to respond to IL-7 by *ex vivo* expansion.

These experiments demonstrated that ShcFFF-expressing pro-B cells were defective in their ability to respond to IL-7, yet I wanted to determine whether placing these cells under a more robust *ex vivo* culture system could overcome this defect by

providing cytokines or cell contact that were not available in previous experiments using growth medium with IL-7. To this end, I plated pro-B cells from *Mb1*-Cre/ShcFFF/*Rag1*$^{-/-}$ mice on OP-9 stromal cells in the presence of 5ng/mL IL-7. However, even in the presence of OP-9 stromal cells, ShcFFF-expressing pro-B cells were unable to respond to IL-7 and displayed severely reduced cell numbers compared with littermate controls (Figure 4-8).

In addition to *Mb1*-Cre/ShcFFF mice, I also placed the *Mb1*-Cre/ShcWT mice on a *Rag1*$^{-/-}$ background. Although expression of ShcWT did not impair B cell development in *Mb1*-Cre/ShcWT mice, I wanted to ensure that it did not affect the ability of pro-B cells to respond to IL-7. Pro-B cells from *Mb1*-Cre/ShcWT/*Rag1*$^{-/-}$ mice were able to respond to IL-7 at all concentrations tested as assessed by increased cell number (Figure 4-9). Interestingly, these cells appeared to have an advantage over littermate controls at higher concentrations of IL-7. This suggests expression of ShcWT may have a positive effect on the ability of pro-B cells to respond to IL-7.

Pro-B cells from *Mb1*-Cre/ShcWT/*Rag1*$^{-/-}$ mice were also able to incorporate [^{3}H]-thymidine at levels comparable to littermate controls (Figure 4-10). These data demonstrate that the capacity of pro-B cells to respond to IL-7 is critically dependent upon the ability of Shc to become phosphorylated on the three tyrosine residues and mediate further downstream signals. Furthermore, this response is independent of the pre-B cell receptor.

Figure 4-5. ***Mb1*-Cre/ShcFFF/*Rag1*$^{-/-}$ mice show decreased pro-B but normal pre-pro-B cell numbers.** Total cell numbers of bone marrow subsets from *Mb1*-Cre/ShcFFF/*Rag1*$^{-/-}$ mice and littermate controls. Numbers were determined from total cell counts and FACS gating. *, $p < 0.01$.

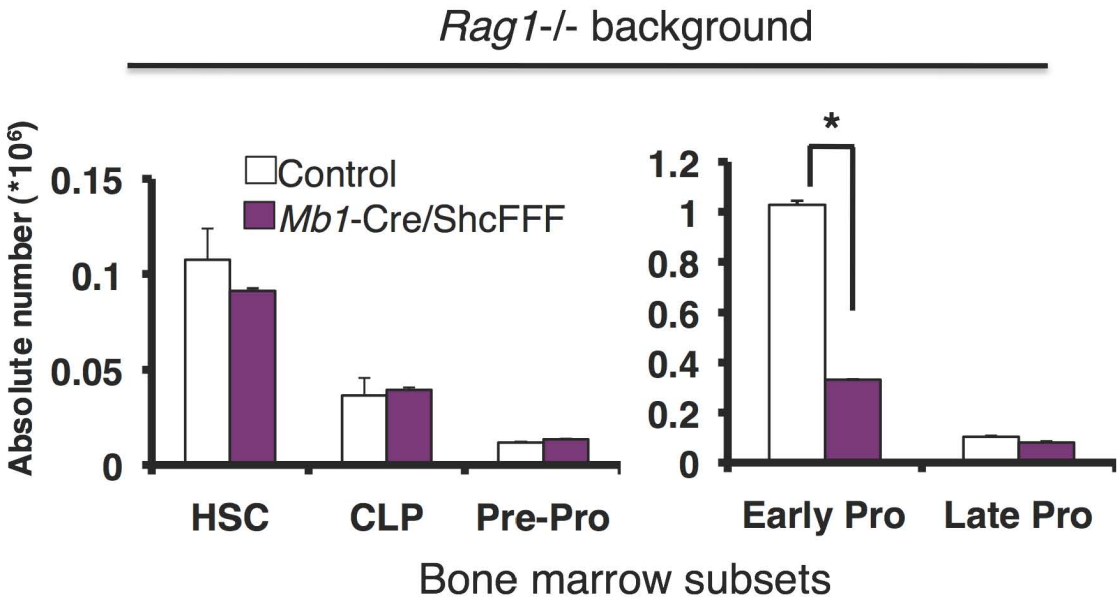

Rag1-/- background
Absolute number (*10^6)
0.15
0.1
0.05
0
Control
Mb1-Cre/ShcFFF
*
1.2
1
0.8
0.6
0.4
0.2
0
HSC
CLP
Pre-Pro
Early Pro
Late Pro
Bone marrow subsets

Figure 4-6. ***Mb1-Cre*/ShcFFF/*Rag1*$^{-/-}$ pro-B cells have normal expression of IL-7Rα**. FACS analysis of CD127 expression on the indicated bone marrow subset and mouse genotype. Multilineage progenitor used as a negative control for IL-7Rα expression.

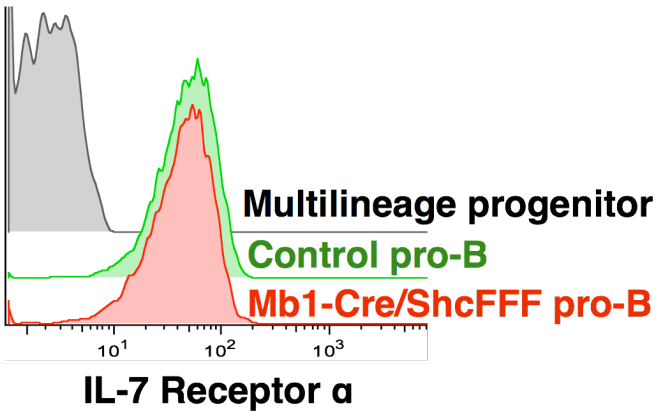

Multilineage progenitor
Control pro-B
Mb1-Cre/ShcFFF pro-B
10^1
10^2
10^3
IL-7 Receptor α

Figure 4-7. **Impaired response of ShcFFF-expressing pro-B cells to IL-7 is independent of the pre-BCR**. $CD19^{+}$ bone marrow cells from the indicated mouse genotype were incubated with varying concentrations of IL-7. ***A***. Cells were pulsed for eight hours with 1 μCi [^{3}H]-thymidine, and [^{3}H]-thymidine uptake was measured. ***B***. Total cell counts were determined with a FACS-based bead assay that included PI to discriminate live cells. Data is representative of two independent experiments.

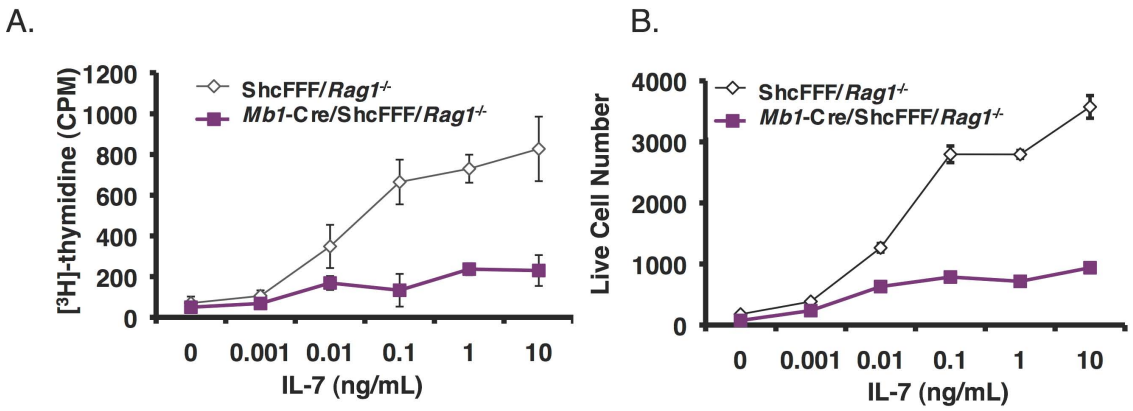

A.
[3H]-thymidine (CPM)
1200
1000
800
600
400
200
0
ShcFFF/Rag1-/-
Mb1-Cre/ShcFFF/Rag1-/-
0
0.001
0.01
0.1
1
10
IL-7 (ng/mL)
B.
Live Cell Number
4000
3000
2000
1000
0
ShcFFF/Rag1-/-
Mb1-Cre/ShcFFF/Rag1-/-
0
0.001
0.01
0.1
1
10
IL-7 (ng/mL)

Figure 4-8. **Culture with OP-9 stromal cells does not rescue defective response of ShcFFF-expressing pro-B cells to IL-7**. $CD19^{+}$ bone marrow cells from the indicated mouse genotype were incubated with 5ng/mL IL-7 on OP-9 stromal cells. On the indicated days, cell number was assessed with a FACS-based bead assay.

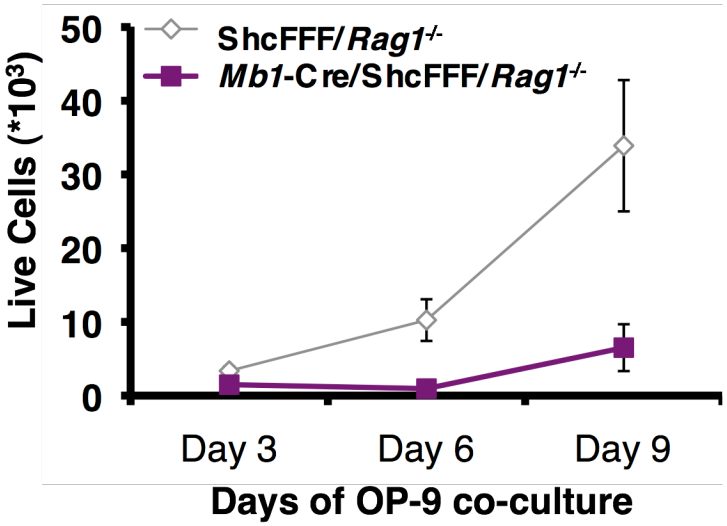

50
40
30
20
10
0
Live Cells (*10³)
ShcFFF/Rag1-/-
Mb1-Cre/ShcFFF/Rag1-/-
Day 3
Day 6
Day 9
Days of OP-9 co-culture

Figure 4-9. **ShcWT-expressing pro-B cells respond normally to culture with IL-7**. $CD19^+$ bone marrow cells from the indicated mouse genotype were incubated with varying concentrations of IL-7. On the fourth day of culture total cell counts were determined with a FACS-based bead assay that included PI to discriminate live cells. Data is from a single experiment.

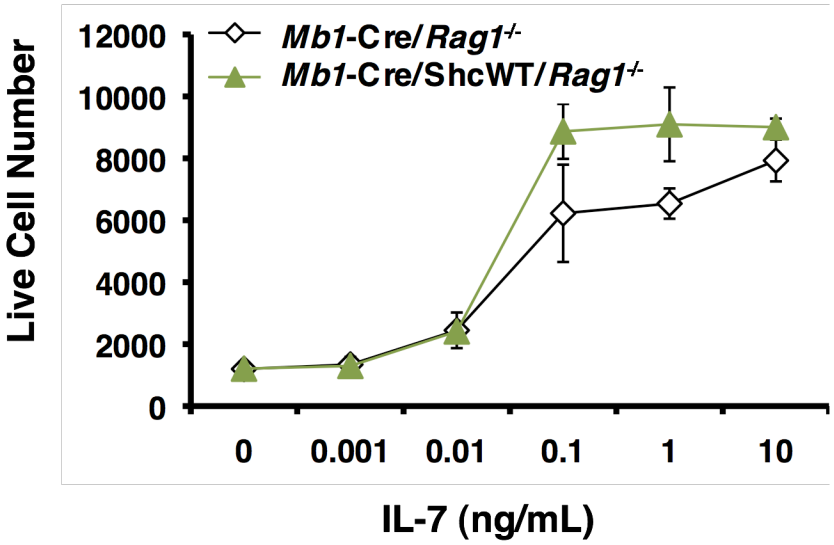
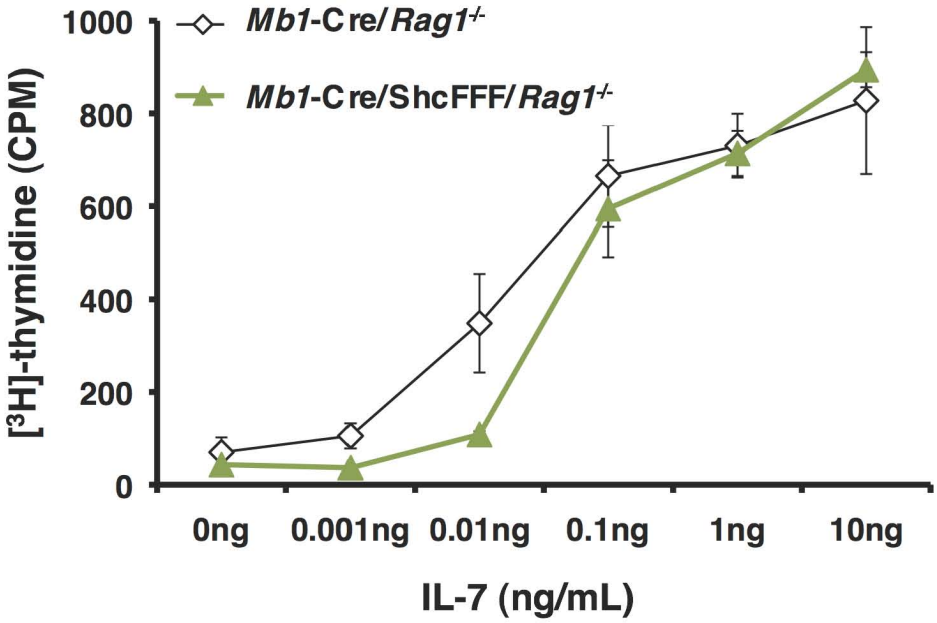

Mb1-Cre/Rag1-/-
Mb1-Cre/ShcWT/Rag1-/-
Live Cell Number
12000
10000
8000
6000
4000
2000
0
0
0.001
0.01
0.1
1
10
IL-7 (ng/mL)

Figure 4-10. ***Mb1*-Cre/ShcWT/*Rag1*$^{-/-}$ pro-B cells incorporate [^{3}H]-thymidine during culture with IL-7**. CD19$^+$ bone marrow cells from the indicated mouse genotype were incubated with varying concentrations of IL-7. On the fourth day of culture, cells were pulsed for eight hours with 1 μCi [^{3}H]-thymidine, and [^{3}H]-thymidine uptake was measured. Data is from a single experiment.


Mb1-Cre/Rag1-/-
Mb1-Cre/ShcFFF/Rag1-/-
[3H]-thymidine (CPM)
1000
800
600
400
200
0
0ng
0.001ng
0.01ng
0.1ng
1ng
10ng
IL-7 (ng/mL)

Mb1-Cre/ShcFFF B cells proliferate normally but have decreased production rates

The previous data demonstrated that ShcFFF-expressing pro-B cells had a defective response to *ex vivo* culture with IL-7. IL-7 can effect multiple responses from a cell, including proliferation, survival, and commitment to the B lineage. Due to the defective proliferation observed in thymocytes from *Lck*-Cre/ShcFFF mice [174], I tested the ability of pro-B cells to respond to IL-7 by proliferation. Sorted pro-B cells from *Mb1*-Cre/ShcFFF mice or littermate controls were labeled with CFSE and cultured in varying concentrations of IL-7 (Figure 4-11). Rather surprisingly, pro-B cells from *Mb1*-Cre/ShcFFF mice proliferated in response to IL-7 at a level comparable to littermate controls. Both the percentage of the population that divided as well as the extent to which they divided (as determined by CFSE dilution) were comparable between *Mb1*-Cre and *Mb1*-Cre/ShcFFF mice. However, the absolute number of cells that showed diluted CFSE were greatly reduced in ShcFFF-expressing cells, which could indicate cell loss.

Although these data suggest that expression of ShcFFF does not impair proliferation of pro-B cells in response to IL-7, I cannot rule out the possibility that the fraction of cells that proliferated did not express ShcFFF (due to likely chance that Cre-mediated recombination of the stop cassette is not 100% efficient in pro-B cells).

As a second means of quantifying proliferation, I pulsed sorted pro-B cells with BrdU during the last 12 hours of culture with IL-7. After staining the cells for surface antigens and then intracellular BrdU incorporation, I gated on pro-B cells and examined the extent of BrdU incorporation (Figure 4-12). Once again, I saw no difference in the percentage of cells that had undergone division (as determined by BrdU incorporation). However, I noticed an increase in the sub-2n gate by 7-AAD (1.44% in control and

5.09% in *Mb1*-Cre/ShcFFF pro-B). This suggested a potential increase in cells that had undergone cell death and DNA degradation. Using two *ex vivo* proliferation assays, I did not detected any difference in the ability of ShcFFF-expressing pro-B cells to proliferate in response to culture with IL-7. However, these analyses suggest a potential defect in the ability of IL-7 to promote survival in *Mb1*-Cre/ShcFFF pro-B cells during *ex vivo* culture.

Next, I wanted to determine if *Mb1*-Cre/ShcFFF mice manifested a defect in proliferation during development in the bone marrow. Continuous BrdU administration is one means of determining population kinetics in the bone marrow. The rate of BrdU incorporation, or the percentage of cells that incorporate BrdU over time, represents the renewal rate of B cell subsets. After gating on pro-B and pre-B cells by FACS, I graphed the percentage of cells that were positive for BrdU over the time of this experiment (Figure 4-13, A). *Mb1*-Cre/ShcFFF did not differ in the renewal rate of pro-B cells (18.5% versus 18.4% in littermate controls) or pre-B cells (19.6% versus 20.5% in littermate controls) compared to *Mb1*-Cre littermates (Table 1).

The total number of $BrdU^+$ cells accumulating over time represents the production rate of bone marrow B cell subsets. FACS analysis including the percentage of $BrdU^+$ cells combined with the total bone marrow cell counts provided the production rate for pro-B and pre-B cells (Figure 4-13, B and Table 1). Pro-B cells from *Mb1*-Cre/ShcFFF mice had a production rate that was decreased by 54% compared to *Mb1*-Cre pro-B cells (37×10^3 cells/day versus 81×10^3, respectively). The production rate of pre-B cells from *Mb1*-Cre/ShcFFF mice was strikingly reduced to only 14% of *Mb1*-Cre pre-B cells (54×10^3 cells/day versus 389×10^3 in controls) (Table 1). This suggested that

Mb1-Cre/ShcFFF bone marrow B subsets were dividing at a rate comparable to littermates. However, they were compromised in the number of cells produced in each subset.

Together these data suggest expression of ShcFFF does not impair the proliferation of early B cells in response to IL-7 stimulation or during the process of B cell development *in vivo*. The decreased production rate of early B subsets *in vivo* demonstrated that, regardless of similar renewal rate, B cell numbers in each subset were not accumulating in *Mb1*-Cre/ShcFFF mice. The increased percentage of cells in the sub-2n gate during *ex vivo* culture suggested a potential defect in survival. Therefore, I sought to investigate the survival of ShcFFF-expressing B cells.

Figure 4-11. **ShcFFF does not impair proliferation in response to IL-7**. FACS sorted pro-B cells from the indicated genotype were cultured in varying concentrations of IL-7. On the fourth day of culture, cells were stained with anti-B220 antibodies, and CFSE dilution of cells from IL-7-containing media (colored line histogram) was plotted against cells cultured in medium alone (shaded gray histogram).

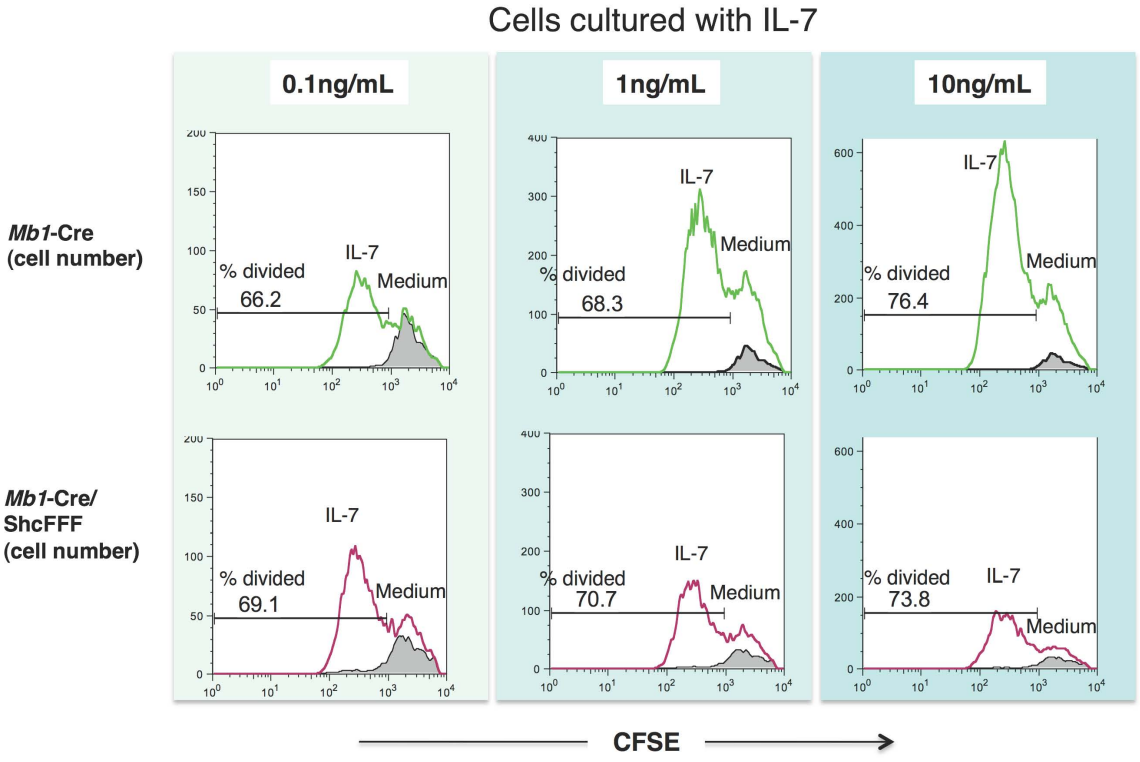

Cells cultured with IL-7
0.1ng/mL
1ng/mL
10ng/mL
Mb1-Cre
(cell number)
Mb1-Cre/
ShcFFF
(cell number)
% divided
66.2
IL-7
Medium
% divided
68.3
IL-7
Medium
% divided
76.4
IL-7
Medium
% divided
69.1
IL-7
Medium
% divided
70.7
IL-7
Medium
% divided
73.8
IL-7
Medium
CFSE

Figure 4-12. **ShcFFF does not disrupt uptake of BrdU during IL-7 culture**. FACS sorted pro-B cells from mice with the indicated genotype were incubated with 1ng/mL IL-7. On the fourth day of culture, cells were surfaced stained with antibodies to detect pro-B cells. Cells were then fixed, permeabilized, and incubated with anti-BrdU antibodies to detect BrdU uptake. 7-AAD was used to identify sub-2n, cells in G_0, and dividing cells.

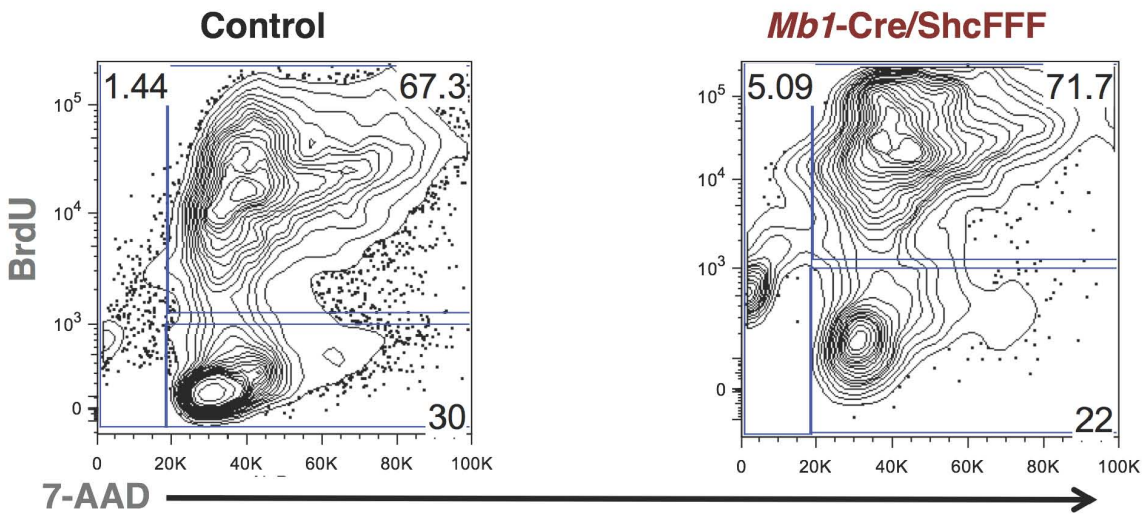
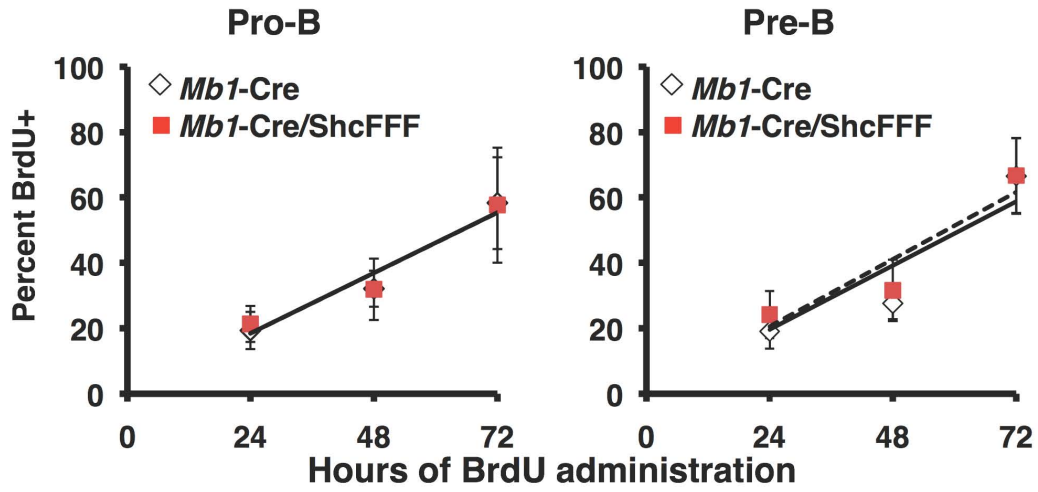

Control
Mb1-Cre/ShcFFF
BrdU
7-AAD
1.44
67.3
30
5.09
71.7
22
0
20K
40K
60K
80K
100K
10^5
10^4
10^3
0

Figure 4-13. **Expression of ShcFFF does not affect cell turnover but decreases production of early B subsets**. Mice from the indicated genotype were given an initial IP injection of BrdU and then continuous BrdU in their drinking water. On the indicated days after the initial BrdU injection, mice were sacrificed. Bone marrow was collected, counted, stained for surface immunophenotyping, and then stained intracellularly for incorporation of BrdU. ***A.*** Percentages of BrdU+ pro-B and pre-B cells over time. ***B.*** Total cell number of BrdU+ pro-B and pre-B cells over time. A minimum of five *Mb1*-Cre and six *Mb1*-Cre/ShcFFF mice were used for each day. Lines indicate the linear regression of *Mb1*-Cre (solid) and *Mb1*-Cre/ShcFFF (dashed) mice. *, $p < 0.01$; **, $p < 0.001$.

A.


Pro-B
Pre-B
Mb1-Cre
Mb1-Cre/ShcFFF
Percent BrdU+
100
80
60
40
20
0
0
24
48
72
Hours of BrdU administration

B.

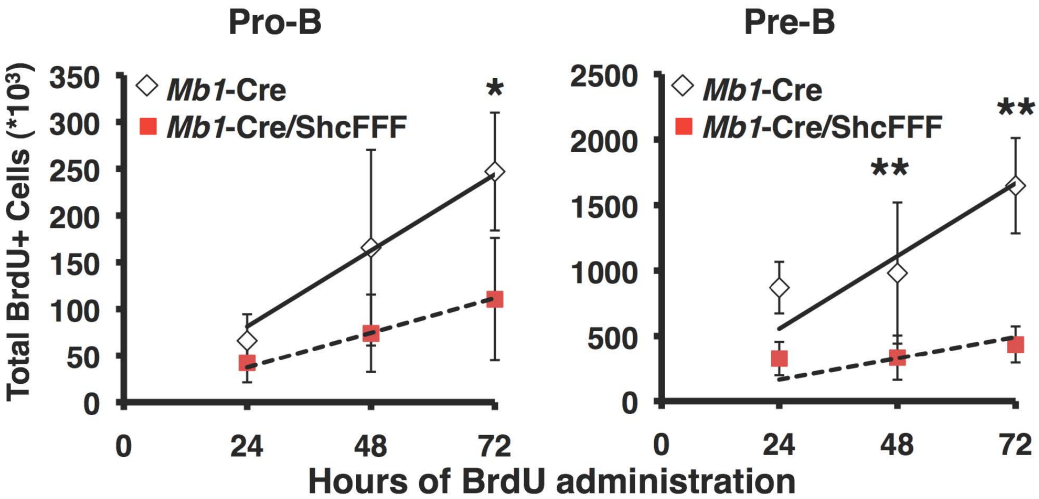

Pro-B
Pre-B
Mb1-Cre
Mb1-Cre/ShcFFF
Total BrdU+ Cells (*10³)
350
300
250
200
150
100
50
0
2500
2000
1500
1000
500
0
*
**
**
0
24
48
72
Hours of BrdU administration

Table 4-1. **Renewal and production rates of bone marrow B cell subsets.** Percentages of $BrdU^+$ pro-B and pre-B cells for each mouse were determined by flow cytometry and multiplied by total bone marrow cell counts (trypan) from two femurs of each mouse to obtain total BrdU+ cell numbers. The regression coefficients of percent and absolute BrdU labeling versus time provide an estimate of renewal and production rates, respectively.

	Renewal Rate (% of pool/day)		Production Rate (cells/day × 10^3)	
	Pro	**Pre**	**Pro**	**Pre**
***Mb1*-Cre**	18.5	19.6	81.3	389.02
***Mb1*-Cre/ShcFFF**	18.4	20.5	37.3	54.016

Mb1-Cre/ShcFFF B cells show indications of increased cell death

To determine if ShcFFF-expressing pro-B cells could be defective in their ability to respond to IL-7 due to an impaired survival, I examined the percentage of PI^+ cells from *Mb1*-Cre/ShcFFF/*Rag1*$^{-/-}$ mice during culture with IL-7 and OP-9 stromal cells (Figure 4-14). Mice on a *Rag1*$^{-/-}$ background were used to eliminate potential differences in differentiation between control and ShcFFF-expressing mice (i.e. impaired pre-BCR signaling-induced cell death) that could affect cell death. After three days of culture with OP-9 stromal cells, *Mb1*-Cre/ShcFFF/*Rag1*$^{-/-}$ pro-B cells showed a 65% increase in PI^+ cells (indicative of late-stage apoptosis) compared to littermate pro-B cells (Figure 4-14). This supported the increased percentage of cells in the sub-2n gate observed in ShcFFF-expressing pro-B cells cultured with IL-7 (Figure 4-12) suggesting that ShcFFF-expressing B cells abnormally undergo apoptosis during culture with IL-7.

The increase in sub-2n cells observed during *ex vivo* culture with IL-7 led me to examine the sub-2n gate in freshly isolated bone marrow subsets. *Mb1*-Cre/ShcFFF mice had an increased percentage of cells in the sub-2n gate beginning at the pro-B stage and continuing to the immature stage. Therefore, ShcFFF expression led to an increase in the sub-2n gate in freshly isolated bone marrow as well as during culture with IL-7.

Finally, I asked whether I might be able to capture cells undergoing apoptosis by FACS analysis of Annexin V from freshly isolated bone marrow. Freshly isolated bone marrow was stained for surface immunophenotyping and then incubated with Annexin V to detect exposure of phosphatidylserine on the cell surface, as part of apoptosis. *Mb1*-Cre/ShcFFF B cells showed increased Annexin V staining beginning at the pro-B stage and continuing through the immature stage (Figure 4-15). The percentage of

Annexin V staining is likely underrepresented for multiple reasons: first, apoptotic cells are generally cleared quite rapidly *in vivo*; second, cell isolation from the bone marrow and cell surface staining required multiple washing and centrifugation steps (potentially losing the more fragile apoptotic cells in the process); yet, *Mb1*-Cre/ShcFFF B subsets clearly showed an increase in surface staining of Annexin V compared to littermate controls.

Together these data demonstrate that an increased percentage of *Mb1*-Cre/ShcFFF B subsets are undergoing apoptosis during development in the bone marrow. Additionally, ShcFFF-expressing pro-B cells appear to abnormally undergo cell death when cultured with the cytokine IL-7.

Figure 4-14. **Increased PI staining in ShcFFF-expressing pro-B cells cultured with IL-7**. $CD19^+$ bone marrow cells from the indicated mouse genotype were incubated on OP-9 stromal cells with 5ng/mL IL-7. After three days of culture, FACS analysis was performed to determine percentage of cells that were positive for PI staining. Data represents the average of PI staining from cells cultured in duplicate wells. **, $p < 0.001$.

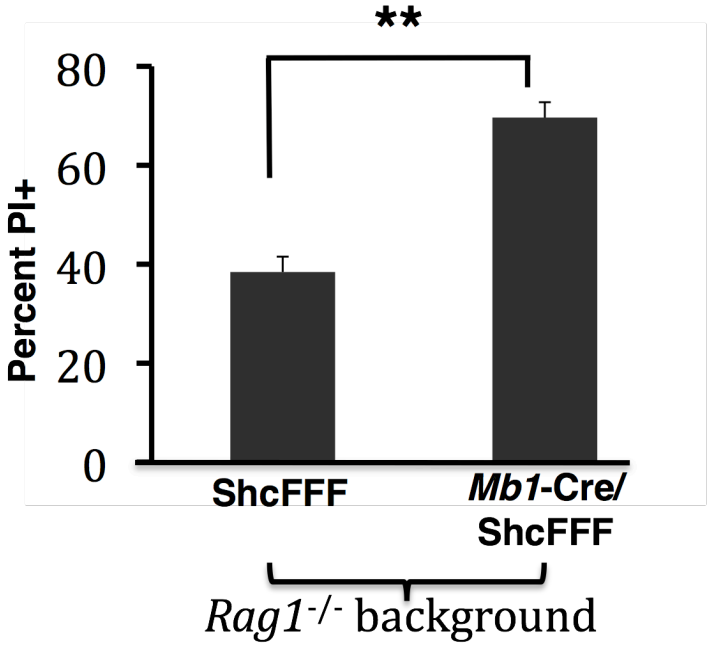

**
80
60
40
20
0
Percent PI+
ShcFFF
Mb1-Cre/
ShcFFF
Rag1-/- background

Figure 4-15. **ShcFFF-expressing bone marrow B cells show increased sub-2n gating**. Freshly isolated bone marrow from the indicated mouse genotype was stained for immunophenotyping. Cells were then fixed, permeabilized, and stained for DNA content. *Mb1*-Cre represents 15 mice; *Mb1*-Cre/ShcFFF represents 19 mice. ***, $p < 0.0001$.

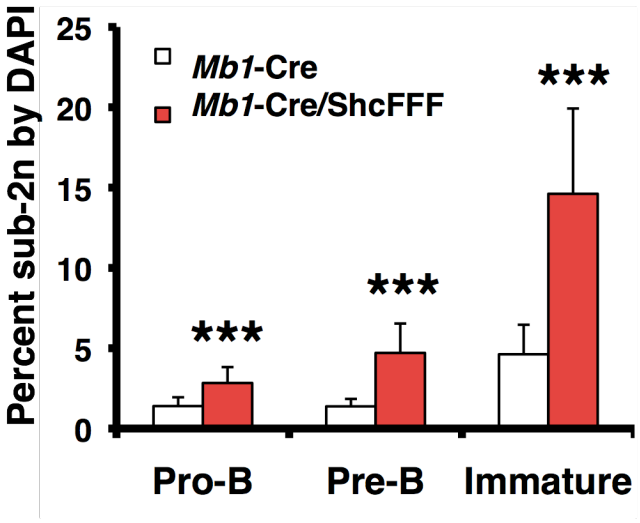
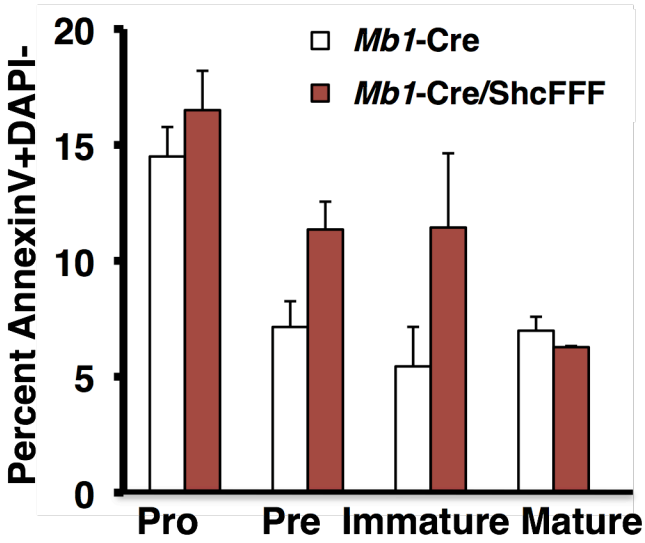

Percent sub-2n by DAPI
25
20
15
10
5
0
Mb1-Cre
Mb1-Cre/ShcFFF
***
***
***
Pro-B
Pre-B
Immature

Figure 4-16. **Increased Annexin V staining in ShcFFF-expressing bone marrow B cells**. Freshly isolated bone marrow was surfaced stained for immunophenotyping and then incubated with Annexin V to detect surface exposure of phosphatidylserine. The percentage of cells staining positive for Annexin V but negative for DAPI are plotted for the indicated bone marrow subsets.


Percent AnnexinV+DAPI-
20
15
10
5
0
Mb1-Cre
Mb1-Cre/ShcFFF
Pro
Pre
Immature
Mature

Discussion

The developmental defects observed in the bone marrow of mice with conditional disruption of Shc signaling could be due to blocks at multiple stages of development. Since a complete understanding of defects in more mature B cells could be confounded by defective signaling during multiple selection checkpoints, I focused on the earliest developmental block in ShcFFF-expressing mice: the pre-pro-B to pro-B transition.

The IL-7 receptor is required for proper B cell development from the pre-pro-B to pro-B transition. However, Shc is not known to conduct signals downstream of this receptor. A $B220^{-}Thy1^{-}$ IL-7-dependent cell line with rearrangement of the μHC (IxN/2B) was transfected with the IL-2 receptor to test the intracellular signaling requirements between the IL-7 and IL-2 receptors [214]. This group noted a higher level of Shc phosphorylation when the cells were stimulated with IL-2 than when they were stimulated with IL-7. This work dismissed Shc as a signaling intermediate in IL-7 signaling. There are several caveats with this experiment, however. First, the developmental stage, or even whether this line is a true B cell line, is not clear. The requirement for certain receptors changes during development, and the activation of intracellular signaling intermediates changes as well. Finally, the reduced level of phosphorylation does not rule out a role for Shc in signal transduction.

Here, I was able to detect inducible phosphorylation of Shc in *Rag1*$^{-/-}$ pro-B cells in response to IL-7 stimulation (Figure 4-1). The source of these pro-B cells is quite important, as I was able to isolate IL-7 receptor stimulation from any influence of the pre-BCR. Phosphorylation of Shc in response to IL-7 receptor stimulation has not been previously reported. This may be due to the fact that the Shc phosphorylation levels I

observed with IL-7 stimulation were lower than I observed during other stimulation events (i.e. crosslinking of the BCR in mature B cells; personal observation). The lower levels of Shc phosphorylation, combined with previous publications, may have prevented others from observing Shc phosphorylation in response to IL-7 stimulation. Shc is often basally phosphorylated in cell lines. Since I used primary cells, rather than cell lines, this likely increased my chances of seeing a change in Shc phosphorylation over basal levels.

Mb1-Cre/ShcFFF B cells showed a defective response to IL-7 stimulation over a range of conditions (Figures 4-3 and 4-4) although they expressed normal levels of the receptor (Figure 4-2, B). This suggests that ShcFFF expression disrupts intracellular signal transmission from the IL-7 receptor rather than expression of the receptor itself.

Expression of the pre-BCR augments the early B cell's response to IL-7 stimulation. Expression of the pre-BCR represents a critical developmental transition during early B cell development. Expression of a μHC on a *Rag2*$^{-/-}$ background increases incorporation of [^{3}H]-thymidine during *ex vivo* culture in low concentration of IL-7 when compared to *Rag2*$^{-/-}$ pro-B cells lacking the μHC [59]. The ability of the pre-BCR to allow [^{3}H]-thymidine uptake in low IL-7 concentrations was further linked to activation of the Erk/MAPK pathway by blocking the effects of pre-BCR expression with different MEK inhibitors [90]. Additionally, activation of Ras and signaling to the Erk/MAPK pathway has been demonstrated to promote differentiation of early pre-B cells to late pre-B cells, which includes exit from the cell cycle and recombination at the *Igκ* light chain locus [58]. This advance in differentiation also renders cells unresponsive to IL-7 stimulation, and cells differentiate in IL-7-containing media to the IL-7 unresponsive state when cultured *ex vivo* [215]. Although Shc has not yet been demonstrated to

participate in pre-BCR-mediated signaling events, Shc is best known to signal to the central Erk pathway.

Disruption of pre-BCR signaling could result in impaired response to low levels of IL-7 or disruption of differentiation to the IL-7-unresponsive late pre-B stage. To remove the caveats of pre-BCR influence on IL-7 responsiveness and differentiation of pro-B cells to an IL-7-unresponsive stage, I bred *Mb1*-Cre/ShcFFF and the *Mb1*-Cre/ShcWT mice on a *Rag1*$^{-/-}$ background. *Mb1*-Cre/ShcFFF/*Rag1*$^{-/-}$ mice displayed decreased pro-B cell numbers (Figure 4-5). This is the same stage as the initial developmental block in *Mb1*-Cre/ShcFFF mice. This finding was important in that it ruled out any possibility of disrupted pre-BCR signaling as a cause for the initial developmental block. Additionally, pro-B cells from *Mb1*-Cre/ShcFFF/*Rag1*$^{-/-}$ mice expressed the IL-7 receptor at levels comparable to *Rag1*$^{-/-}$ littermate controls (Figure 4-6).

Mb1-Cre/ShcFFF/*Rag1*$^{-/-}$ pro-B cells showed an even more dramatic defect in their ability to respond to IL-7 as measured by [^{3}H]-thymidine incorporation and live cell counts than mice on a plain C57Bl/6 background (Figures 4-3 and 4-7). This disrupted response to IL-7 was not rescued by placing the pro-B cells on a more robust *ex vivo* culture system with OP-9 stromal cells (Figure 4-8). Littermate control pro-B cells continued to show increased cell number after nine days of culture whereas ShcFFF-expressing *Rag1*$^{-/-}$ pro-B cell numbers remained dramatically reduced. This demonstrated that the inability of ShcFFF-expressing pro-B cells to respond to IL-7 was not due to disrupted pre-BCR signaling. In fact, loss of the pre-BCR dramatically

enhanced the difference in cell number and [^{3}H]-thymidine incorporation between control and ShcFFF-expressing pro-B cells during *ex vivo* assays.

Considering the recent understanding of the requirement for the pre-BCR in allowing differentiation of early B cells from an IL-7-dependent to an IL-7-unresponsive stage, it is interesting to consider the possibility that *Mb1*-Cre/ShcFFF mice were defective in signaling through the pre-BCR. This would prevent them from advancing to an IL-7-unresponsive stage and may have resulted in a higher proportion of cells from the ShcFFF-expressing mice remaining at an IL-7-dependent stage. Then, when placing both control and ShcFFF-expressing mice onto a *Rag1*$^{-/-}$ background and removing the ability to differentiate, the control cells remained in the pro-B stage and surpassed the ShcFFF-expressing cells in their response to IL-7 stimulation. Another possibility is that the pre-BCR may have aided signaling through the IL-7 receptor in *Mb1*-Cre/ShcFFF mice, thereby aiding their response to IL-7. When the pre-BCR was removed, this could have rendered the ShcFFF-expressing mice unable to respond to IL-7. These two possibilities could implicate or negate a role for Shc in signaling through the pre-BCR.

Importantly, *Mb1*-Cre/ShcWT/*Rag1*$^{-/-}$ mice were not impaired in their ability to respond to IL-7 (Figures 4-9 and 4-10). This suggests that the impaired ability of ShcFFF-expressing pro-B cells to respond to IL-7 is due specifically to the loss of the three critical tyrosine residues. *Mb1*-Cre/ShcWT/*Rag1*$^{-/-}$ pro-B cells increased in cell number as well as or better than littermate controls in response to *ex vivo* culture with IL-7. Since mice on a *Rag1*$^{-/-}$ background are incapable of differentiating beyond the pro-B stage, this suggests that Shc may play a role in proliferation, survival, or commitment to the B lineage in response to IL-7 stimulation.

I did not detect impaired IL-7-mediated proliferation using CFSE dilution or BrdU incorporation during *ex vivo* culture, although ShcFFF-expressing cells showed decreased cell number when compared to control during *ex vivo* culture with IL-7 (Figures 4-11 and 4-12). Therefore, it seems unlikely that Shc is impeding IL-7-mediated proliferation. In addition, expression of ShcFFF did not affect the renewal rate of pro-B or pre-B cells during normal *in vivo* lymphopoiesis (Figure 4-13 and Table 4-1). The consistency of *in vivo* and *ex vivo* data further support a role for Shc in IL-7 signaling during B cell development. *Mb1*-Cre/ShcFFF pro-B cells showed an increase in the sub-2n DNA gate during *ex vivo* culture in IL-7. Cells located in the sub-2n gate by FACS are likely undergoing DNA degradation as a result of death.

Cells that stain positive for the DNA-binding dyes PI or DAPI have lost their membrane integrity, and this is indicative of late-stage apoptosis. Expression of ShcFFF resulted in increased PI staining of pro-B cells during *ex vivo* culture with IL-7 (Figure 4-17). In addition, I saw statistically significant increases in the sub-2n DNA gate of early B subsets in freshly isolated *Mb1*-Cre/ShcFFF bone marrow. This data correlates nicely with the increased percentage of sub-2n DNA-containing cells in ShcFFF-expressing pro-B cells during *ex vivo* culture with IL-7.

Staining with DNA dye and presence of a sub-2n DNA population are both indicative of late-stage apoptosis. Supporting the possibility of ShcFFF-expressing B cells undergoing apoptosis, early B subsets showed an increase in staining for Annexin V. Interestingly, the mature, recirculating B cells did not display an increase in Annexin V staining. These subsets do not differ significantly in *Mb1*-Cre/ShcFFF mice when compared to control mice, thus it is not surprising that they do not stain highly for

Annexin V. However, the presence of increased Annexin V staining on pre-B cells and immature B cells could suggest additional roles for Shc during additional stages of B cell development in the bone marrow.

Taken together, these data suggest that expression of ShcFFF blocks early B cell development due to increased cell death beginning at the pro-B stage. ShcFFF expression does not appear to impair proliferation. The similarity of defects observed during *ex vivo* culture with IL-7 with cells from freshly isolated bone marrow suggest that defective response to IL-7 may contribute to the developmental block observed in *Mb1*-Cre/ShcFFF mice.

Chapter V

Summary and Future Directions

In this chapter, I review some of the significant observations from chapters 3 and 4. I attempt to highlight the significance of these findings and how they relate to current understanding of Shc and B cell development. I also discuss potential future directions for this project in furthering our understanding of Shc signaling and early B cell development

Novel requirement for Shc in B cell development

In chapter III, I identified a developmental block in early B cells isolated from *Mb1*-Cre/ShcFFF mice. This finding is significant for three reasons: First, it reveals a novel requirement for Shc during B cell development. These mouse models contribute to the understanding of lymphopoiesis in the bone marrow and provide models for further testing of Shc function in B cells. Second, I demonstrate a requirement for Shc during B cell development that is independent of the pre-BCR. This is at an earlier developmental stage than identified during T cell development. Third, findings from 8 to 12 week-old *Cd19*-Cre/ShcFFF and *Mb1*-Cre/*Shc*$^{f/f}$ mice suggest that Shc is required for B cell development specifically during early developmental stages. Therefore, the significance of Shc signaling is not constant throughout B cell development. Rather, it plays a non-redundant role during early developmental events.

Targeted disruption of Shc for in vivo studies. The Cre/*loxP* system provides an enormous advantage for the study of conditional knockout genes. *Shc1* knockout mice are embryonic lethal, and expression of ShcFFF in place of endogenous *Shc1* disrupts motor coordination and reduces the percentage of these pups represented in each litter [136, 137]. Conditional targeting of *Shc1* deletion or expression of ShcFFF has allowed me to specifically probe a cell-intrinsic Shc function during B cell development. This creates an extremely exciting system in that I am able to test the requirement for Shc during the multiple stages and requirements of development within the normal bone marrow microenvironment. This system has allowed me to address the biological relevance of the Shc protein without restricting its relevance to specific receptors, stages of development, or effector functions (i.e. proliferation or survival). Use of conditional models prevented cell-extrinsic factors due to impaired Shc signaling from confounding the role of Shc during B lymphopoiesis. In addition, these models induced Cre-mediated recombination during specific stages of B cell development. By using different Cre transgenic mouse lines, I have tested the significance of Shc during different stages of B cell development and found that the timing of targeted Shc disruption is important to observe a phenotype. The biological significance of Shc lies during early developmental stages rather than later. It remains to be determined if Shc could play additional roles at stages of lymphocyte development prior to the CLP stage.

Although the Cre/*loxP* system I utilized is an exceptional system for this study, it has certain caveats. First, conditional expression of the transgene or conditional loss of the endogenous *Shc* genomic sequence is dependent upon expression of the Cre recombinase and its efficacy of recombination. One should always consider the

possibility that a normal response may be due to lack of Cre-mediated recombination in that subset. In my system, this could be addressed by transfection of *Rag1*$^{-/-}$ pro-B cells with ShcFFF in a GFP reporter construct. This would allow clear distinction of the ShcFFF-expressing pro-B cells in comparison to normal pro-B cells.

A particularly difficult aspect of this project was that recombination at the *loxP* sites occurred in a rare population of cells in the bone marrow. Furthermore, expression of the ShcFFF protein is low compared to endogenous Shc and very close to p52 Shc in size. The high levels of p52 Shc relative to ShcFFF as well as their nearly identical size obscured the ShcFFF protein in total cell lysates, and I was only able to detect it through immunoprecipitation with anti-FLAG antibodies from total CD19^{+} bone marrow or spleen cells. I was also unable to detect ShcFFF in Western blots of total cell lysates with anti-FLAG. The block in B cell development at the pre-pro-B to pro-B stage in *Mb1*-Cre/ShcFFF mice as compared to *Mb1*-Cre littermates suggests the ShcFFF protein is present and disrupting B cell development. However, I cannot rule out the possibility that Shc may play a role at the CLP to pre-pro-B transition since I cannot prove in this system that the ShcFFF protein is present.

The dramatic phenotype observed in *Mb1*-Cre/ShcFFF mice is impressive considering the relatively low abundance of ShcFFF protein compared to endogenous Shc. There are several potential explanations for this phenomenon. First, Shc may function near the initiation of a signaling cascade. Thus, low levels of the dominant negative protein may be sufficient to disrupt this process. Another possibility may be that ShcFFF-expressing cells are underrepresented in the bone marrow of *Mb1*-Cre/ShcFFF mice. ShcFFF-expressing cells may be eliminated during

development. Since the *Mb1*-Cre drives Cre expression throughout B cell development, recombination could continue as cells develop to a stage that is less dependent upon Shc signaling. This could explain the presence of ShcFFF protein in splenic B cells even if cells without Cre-mediated recombination dominated the bone marrow. Finally, the low level of ShcFFF protein may be a combined effect of the low avidity of the anti-FLAG antibody with the presence of a single FLAG tag on the ShcFFF protein. This could lead to a problem of inefficient detection by Western blotting techniques rather than extremely low abundance of the ShcFFF protein.

Real-time PCR is sensitive enough to detect single base pair substitutions in RNA sequences while requiring as little as one cell for the template. In order to detect expression of the ShcFFF in early B subsets, probes against the FLAG sequence or a region including the Y to F mutations in the CH1 region could detect the ShcFFF sequence in early B subsets. This could provide a means of determining if the ShcFFF protein is present in the rare populations of the bone marrow.

Shc is required specifically during early B cell development. A very interesting finding in this study is the specificity for Shc requirement during early B cell development. Cre-mediated recombination during later stages of B cell development resulted in no apparent defect in B cell development. This finding is supported by two different mouse crosses.

First, *Cd19*-Cre mediates recombination at the *loxP* sites beginning at the pro-B cell stage, yet often full recombination (and, in the instance of conditional gene knockout, subsequent loss of protein) is not observed until the pre-B cell stage and later [116, 178].

Whereas *Mb1*-Cre/ShcFFF had diminished cellularity beginning with the pro-B population, *Cd19*-Cre/ShcFFF mice did not show any developmental defect. In *Lck*-Cre/ShcFFF mice, recombination efficiency of the ShcFFF transgene in the DN subest is only around 30%, and these mice show a severe developmental block at the DN3 to DN4 transition [174]. Furthermore, over 80% of the DN subset is arrested at the DN3 stage, suggesting that the majority of the DNA for the Southern blot analyses came from the DN3 subset. Total $CD19^{+}$ bone marrow DNA from *Cd19*-Cre/ShcFFF mice shows around 46% recombination in the bone marrow and 70% recombination in the spleen. This suggests two things: first, Cre-mediated recombination occurs in the bone marrow B cells of *Cd19*-Cre/ShcFFF mice, and recombination occurs at a level comparable or better than *Lck*-Cre/ShcFFF mice, which show a dramatic phenotype in T cell development; second, B cells that have deletion of the STOP cassette are not lost since the efficiency of recombination is even greater in the spleen and the ShcFFF protein is detected. This supports a hypothesis that conditional expression of ShcFFF after a certain stage of B lymphopoiesis does not disrupt later developmental events.

The second mouse line supporting the requirement of Shc during early B cell development is the eight-week-old *Mb1*-Cre/$Shc^{f/f}$ mice. Shc protein is quite stable with a relatively long half-life of around 24 hours (personal communication, Paul Trampont, determined through a pulse-chase assay for Shc half-life). Although *Mb1*-Cre directs Cre-mediated recombination beginning with at the CLP stage, I detected rather high levels of Shc protein in pro-B cells. Endogenous Shc was efficiently cleared at the pre-B stage, yet these mice displayed no developmental abnormality in the observed B cell subsets. However, when I allowed these mice to age to nine months, I observed a defect

in B cell development that mirrored *Mb1*-Cre/ShcFFF mice. Senescence can allow more subtle defects in B cell development to become apparent, as production and differentiation rates slow in aged mice. This could allow Shc protein to be deleted early enough to have an effect on development. This further supports a requirement for Shc specifically during early B cell developmental stages.

The requirement for Shc specifically during the early stages of lymphocyte development is supported by my own unpublished data. Crossing ShcFFF transgenic mice with mice transgenic for the distal *Lck*-Cre (*dLck*-Cre) permits Cre recombination during the late DP stage of thymocyte development [216]. In contrast to proximal *Lck*-Cre/ShcFFF mice, *dLck*-Cre/ShcFFF mice display completely normal numbers of DN (DN1 – DN4), DP, $CD4^+$ SP, and $CD8^+$ SP thymocytes in the thymus as well as normal numbers of $CD4^+$ and $CD8^+$ cells in the spleen (personal observation). In addition, these cells proliferate normally (as assessed by CFSE dilution) in response to anti-CD3 + anti-CD28 stimulation. Peripheral T cells show 60% recombination efficiency in the ShcFFF transgene by Southern blot, demonstrating that lack of recombination is an unlikely explanation for the ability of these cells to develop or respond to stimulation through the T cell receptor. These findings were complimented with *Ox40*-Cre/ShcFFF mice, in which Cre-mediated recombination is driven in activated peripheral T cells. These mice also display normal T cell development as well as normal capability to respond to anti-CD3 + anti-CD28 crosslinking (as assessed by CFSE dilution) (personal observation). Together, the *dLck*-Cre/ShcFFF and *Ox40*-Cre/ShcFFF mice suggest that expression of ShcFFF during stages of T lymphocyte development after

the pre-TCR checkpoint *in vivo* does not impede successive stages of T cell development or capacity of peripheral T cells to respond to stimulation through the TCR.

Published data further supports a requirement for Shc specifically within less differentiated cell types. Examination of various human CML lines reveals variations in both the level of SHC protein expressed as well as its phosphorylation status. SHC protein is highly expressed in $CD34^+$ CML lines as well as $CD34^+$ bone marrow samples but barely detectable in $CD34^-$ CML and $CD34^-$ bone marrow [217]. (CD34 is a marker for early B subsets.) In addition, SHC is highly phosphorylated in $CD34^+$ CML lines but not in $CD34^-$ CML lines. A similar correlation occurs in the developing mouse brain. Shc is highly expressed in immature, rapidly diving cells of the brain. However, expression of ShcA protein declines to nearly undetectable levels during neuronal differentiation [218]. Together, these data suggest that expression of Shc protein is correlated with the status of differentiation. Therefore, the significance of Shc in signal transduction may wane as cell progress toward more terminally differentiated stages.

A new role for Shc in IL-7-mediated survival

Due to the stage at which B cell development was blocked in *Mb1*-Cre/ShcFFF mice, the list of possible receptors with disrupted signaling was narrowed considerably. IL-7 and its cognate receptor play critical roles in B lymphopoiesis, particularly at the pre-pro-B to pro-B transition [95]. Other proteins that have been shown to play roles at this stage include E2A, NFκB, STAT3, and STAT5 [212, 219-221]. Of these proteins, only the STAT family members are activated proximal to the IL-7 receptor. Interestingly, Shc, STAT3, and STAT5 all play roles downstream of other cytokine

receptors, including the IL-2 receptor [222]. Therefore, although other proteins had been linked to conducting intracellular signals from the IL-7 receptor, I felt it remained likely that Shc could play a separate important role during IL-7R signal transduction.

In chapter IV, I further examine the developmental block in *Mb1*-Cre/ShcFFF mice and identify a deficiency of pro-B cells from these mice to respond to IL-7. Within this chapter I make two significant findings: First, I isolate a deficient response of ShcFFF-expressing pro-B cells to respond specifically to IL-7 stimulation. This receptor has not been previously linked to signaling through the Shc adaptor protein. Second, I used a variety of approaches to separate the potential defects in cellularity in *Mb1*-Cre/ShcFFF B cells and find that expression of ShcFFF is leading to abnormal apoptosis both *in vivo* and during *ex vivo* culture with IL-7.

Current understanding of IL-7 receptor signaling and the role of Shc: Current understanding of the IL-7R firmly implicate the STAT family of proteins in signal transduction with IL-7 stimulation. Yet, the IL-7 receptor potentiates multiple cellular responses, including proliferation, differentiation, survival, and commitment to the B lineage. Due to the pleiotropic effects of IL-7 receptor signaling, it was possible that Shc could play a role downstream of this receptor in mediating one of these cellular responses. As I learned more about IL-7 receptor signaling, I noticed several papers did not fully distinguish between survival and proliferation of cells during IL-7 culture. For example, decreased [^{3}H]-thymidine incorporation or failure of *ex vivo* cells to expand in number during culture with IL-7 was reported as defects in proliferation. However, these assays cannot distinguish between defects in proliferation, survival, or loss of IL-7

responsiveness due to differentiation or change in lineage commitment. STAT5 is implicated in B lineage commitment, as the *Pax5* promoter contains a STAT-binding motif that interacts with STAT5 and EBF during IL-7 stimulation [211]. Thus, in early B cells, STAT5 may play an important role in maintaining the B lineage [95]. Although this does not exclude it from other effects of IL-7 stimulation, it certainly leaves the door open for Shc in contributing toward IL-7 signal transduction.

By placing the *Mb1*-Cre/ShcFFF mice onto a *Rag1*$^{-/-}$ background, I efficiently removed potential confounding influences of differentiation and signaling through the pre-BCR. However, it should be noted that this does not exclude a role for Shc in these processes. Shc could still play a role in differentiation from the pre-pro to pro-B stage or during earlier stages of B cell development. Expression of ShcFFF did not affect expression of the IL-7 receptor. Expression of the IL-7 receptor on ShcFFF-expressing pro-B cells from mice on a C57Bl/6 or *Rag1*$^{-/-}$ is comparable to littermate controls.

The inability of ShcFFF-expressing pro-B cells to respond to IL-7 was initially revealed as decreased uptake of [^{3}H]-thymidine. However, this method is unable to clearly identify the defective cellular response. Therefore, I measured the ability of ShcFFF-expressing pro-B cells to proliferate in response to IL-7 with two methods: 1) incorporation of BrdU and 2) dilution of CFSE during *ex vivo* culture with IL-7. I saw no difference in the ability of ShcFFF-expressing pro-B cells to proliferate in response to IL-7 as assessed with these methods. Furthermore, *in vivo* analysis of BrdU incorporation demonstrates that *Mb1*-Cre/ShcFFF early B subsets incorporate BrdU at a similar rate as *Mb1*-Cre littermates. Therefore, expression of ShcFFF does not impair

proliferation of early lymphocytes during *in vivo* development or *ex vivo* culture with IL-7.

IL-7 is intimately connected with commitment to the B lineage by regulating the transcription factors *EBF* and *Pax5*. Therefore, it is necessary to address commitment to the B lineage in ShcFFF-expressing cells with potentially compromised IL-7 responses. I allowed ShcFFF-expressing pro-B cells to grow on OP-9 stromal cells in the presence of IL-7, and after nine days of culture, all cells maintained the B lineage as assessed by expression of CD19. Furthermore, I have not noticed any loss of B lineage markers during shorter incubation times with IL-7 in ShcFFF-expressing pro-B cells. However, this experimental setup lacked additional cytokines, such as SCF and Flt3 ligand that could permit expansion of non-B lineage cells. A more detailed analysis is required to determine if Shc plays a role in B lineage commitment at the pro-B stage. These analyses could include detection of B cell-specific genes, including those regulated by the Pax-5 transcription factor that maintain B lineage commitment.

Since I had removed the ability of pro-B cells to differentiate (by placing on a *Rag1*$^{-/-}$ background), and data suggested that expression of Shc did not impair proliferation during culture with IL-7, I examined the survival of pro-B cells from *Mb1*-Cre/ShcFFF mice. I used multiple FACS techniques as indicators of apoptosis. These analyses suggested that ShcFFF-expressing pro-B cells are undergoing an increased rate of apoptosis as identified by: 1) Increased percentage of ShcFFF-expressing cells staining positive for propidium iodide after culture with IL-7; 2) Increased percentage of ShcFFF-expressing cells located within the sub-2n gate both after culture with IL-7 as well as freshly isolate bone marrow; 3) Increased staining with

Annexin V of freshly isolated bone marrow B cells from *Mb1*-Cre/ShcFFF mice compared to littermate controls. Together, these analyses suggest that ShcFFF-expressing pro-B cells are undergoing an abnormally high rate of apoptosis both during normal *in vivo* development as well as with *ex vivo* culture with IL-7. Furthermore, the inability of ShcFFF-expressing pro-B cells to respond to IL-7 *ex vivo* may explain the developmental block in *Mb1*-Cre/ShcFFF B cells.

Current understanding of Shc and survival: In addition to its roles in proliferation and differentiation, Shc has also been studied for its ability to promote survival. In the following paragraphs I have summarized some of the papers I feel are most pertinent to my studies presented here.

The Ba/F3 pro-B cell line is dependent upon IL-3, and withdrawal of this cytokine rapidly induces cell death. Transfection of Ba/F3 cells with the EGFR allows cells to proliferate and survive in low levels of IL-3 (levels that would not normally permit survival) in response to EGF stimulation. Expression of EGFR kinase-defective mutants (V741G or Y740F) blocks the increase in cell number observed with EGF stimulation, yet Ba/F3 expressing these mutant EGFRs maintain a constant cell number over several days [223]. In addition, these receptors induce Shc phosphorylation upon EGF stimulation as well as Ras-GTP loading, B-Raf activation, and MAPK phosphorylation. Interestingly, the K721R EGFR kinase-defective mutant loses cell number during incubation with EGF. Furthermore, these cells fail to induce Shc phosphorylation, B-Raf activation, or MAPK phosphorylation in response to EGF stimulation. These data implicate Shc and the Ras/MAPK pathway to survival during EGF stimulation.

Similarly, signaling through the GM-CSF receptor can induce proliferation as well as survival. Abrogation of the Ras/Raf-1/MAPK pathway fails to prevent cell death in response to GM-CSF stimulation, yet proliferation and DNA synthesis proceed normally [224].

Finally, studies using the same ShcFFF and *Shc*$^{f/f}$ mice as used here demonstrate a requirement for Shc and Shc phosphorylation in apoptosis. Mice expressing Cre recombinase under the control of the *Nestin* promoter and neural specific enhancer target conditional expression of ShcFFF (in ShcFFF transgenic mice) or conditional deletion of *Shc1* (in *Shc*$^{f/f}$ mice) in developing neural progenitors. Disruption of Shc signaling results in decreased brain size, although this phenotype is more severe in ShcFFF-expressing mice than in mice with conditional deletion of *Shc* (perhaps as a result of delayed loss of Shc protein after Cre-mediated recombination at the *Shc1* locus). The reduction in brain size is due to increased apoptosis of neural progenitors during embryonic brain development rather than impaired proliferation or premature neuronal differentiation [225].

Together, these data provide evidence for Shc in regulating or preventing apoptosis of immature cells. Additionally, these data implicate phosphorylation of Shc as a requirement for survival. This is demonstrated by 1) loss of Shc phosphorylation leading to apoptosis, and 2) expression of the dominant negative ShcFFF protein leading to apoptosis. It is intriguing to consider the possibility that disrupted signaling from Shc to the Ras/MAPK pathway could account for the increased apoptosis in *Mb1*-Cre/ShcFFF mice.

Future directions

What mechanism causes abnormal apoptosis in ShcFFF-expressing B cells? The data presented here do not directly address or provide direct evidence for a disrupted signaling pathway in ShcFFF-expressing cells. To determine the mechanism underlying the phenotype of increased apoptosis due to impaired Shc-mediated signaling, I feel that it is logical to begin by testing direct causes of apoptosis. The Bcl-2 family members regulate the mitochondrial pathway of apoptosis. Variations in Bcl-2 levels have been noted upon withdrawal [105] or overexposure [106] of cells to IL-7. However, the significance of Bcl-2 levels during IL-7 withdrawal in pro-B cells has been questioned, which leaves the door open for other Bcl-2 family members in regulating apoptosis of early B cell subsets [107].

I performed real-time PCR to examine the *Bcl-2* levels in bone marrow B cells. Lower levels were observed in cells with high rates of apoptosis (pro-B and pre-B), and the highest levels were expressed in the long-lived mature, recirculating B cell population. However, *Mb1*-Cre/ShcFFF mice expressed levels of *Bcl-2* comparable to littermate controls in all populations tested. This finding could imply that expression of ShcFFF does not affect levels of *Bcl-2* or that cells with lower *Bcl-2* levels were lost (perhaps during the rather long process of FACS sorting) and/or are underrepresented in this experiment. The first possibility suggests that apoptosis may be induced by another apoptosis-regulating protein, and the second possibility could be due to missing a critical stage of apoptosis induction.

Several tools are available to address the possibility that proteins other than Bcl-2 are mediating the abnormal rate of apoptosis in *Mb1*-Cre/ShcFFF mice. Real-time PCR probes are readily available for other Bcl-2 family members, including Bcl-xL, Bim, Bax, and Mcl-1. Antibodies to detect active caspases by Western blotting or FACS analysis are also readily available and can provide clear answers as to the activation status of certain caspases. If the pathway inducing apoptosis can be identified, it would provide a solid ending point from which to begin working upward in the direction of Shc signaling.

If one is unable to detect changes in *Bcl-2* levels due to the loss of cells undergoing apoptosis, it may be beneficial to place cells in an *ex vivo* culture in order to retain dead and dying cells. One way to detect activation of caspases in living cells would be to use the PhiPhi-Lux fluorogenic apoptosis assay substrate during *ex vivo* culture of pro-B cells with IL-7. This system is advantageous because it allows the detection of caspase 3 and caspase 3-like activities in living cells by using a cell-permeable substrate that only fluoresces when cleaved at a cleavage recognition site for caspases 3. Moreover, detection of caspases 3 activity is an indication of upstream events, including loss of mitochondrial membrane integrity leading to release of cytochrome *c* and activation of caspases 9.

Considering Ras and the MAPK pathway in apoptosis: Signaling from Shc to Ras is clearly established, and this pathway is linked to activation of the Erk/MAPK pathway. Furthermore, disruption of Ras signaling demonstrates a requirement for proper Ras signaling during the pre-pro-B to pro-B developmental stages. Thus, defective signaling from Shc to Ras must be considered as a potential mechanism leading

to the defect observed in *Mb1*-Cre/ShcFFF mice. I feel this prompts two questions: 1) Is Shc the dominant player in conducting signals to the Ras/MAPK pathway, or 2) Is Shc important in mediating a correct response, i.e. does Shc provide important spatiotemporal regulation of the Ras/MAPK response?

The first question could be quickly addressed in *Mb1*-Cre/ShcFFF/*Rag1*$^{-/-}$ pro-B cells. Activation of Erk in response to IL-7 stimulation of pro-B cells can be efficiently detected with either Western blotting or FACS analysis. Any change in Erk activation, including a change in phosphorylation intensity, kinetics of Erk activation, or duration of the response, would all be helpful in understanding the signaling mechanism underlying the defective ability of ShcFFF-expressing pro-B cells to respond to IL-7. These data would also have implications for the second question regarding the requirement for Shc in the spatiotemporal regulation of the Ras/MAPK response.

A potential role for Shc in c-Abl signaling during B lymphopoiesis: Due to the pleiotropic effects with Shc phosphorylation, I hypothesized that proteins in addition to Grb2 may bind phosphorylated Shc. To identify potential binding partners, I created a modified yeast two-hybrid assay with the phosphorylated CH1 region of Shc as bait. Using this assay, I identified multiple SH2-containing proteins that interacted with phosphorylated Shc, including Grb2 (discussed in Appendix I below). Two proteins identified that were of particular interest were the Abl family members, c-Abl and Arg. The role of c-Abl is rather complex due to its opposing effects based upon its localization. Nuclear c-Abl appears to induce cell death by apoptosis whereas cytoplasmic c-Abl promotes cellular proliferation.

Although c-Abl protein is present throughout development, the level of c-Abl phosphorylation is greatest in the pro-B cell population, suggesting a functional role during this stage of B lymphopoiesis [226]. Mice with targeted disruption of *c-Abl* show cell-intrinsic defects in B cell development [227, 228]. *c-Abl*-deficient mice possess >50% reduction in pro-B cell numbers, and pre-B cells are even further underrepresented [229]. Furthermore, these subsets show elevated levels of apoptosis during *ex vivo* culture without loss of cell cycle progression as well as an increased sensitivity to IL-7 withdrawal [229, 230].

Phosphorylated Shc may provide a docking site for c-Abl during IL-7 signaling, thereby preventing the apoptotic effects of c-Abl in the nucleus. This interaction could also allow c-Abl to exert positive effects by its activity in the cytoplasm. Pro-B cells from c-*Abl*-deficient mice show an accelerated rate of death upon IL-7 withdrawal, although they can proliferate and differentiate during culture with IL-7 [230]. This increase in apoptosis while maintaining proliferation and differentiation is similar to the phenotype in ShcFFF-expressing pro-B cells. It is interesting to consider the possibility that Shc and c-Abl may function together to allow cells to survive in response to the essential cytokine IL-7.

In conclusion, the data presented here highlight a previously unknown role for Shc during B lymphopoiesis. This role is linked to a survival response to the cytokine IL-7. Yet, additional data are required to fully understand how Shc functions in this novel role. First, it is necessary to establish the molecular pathways involved in signal transduction from phosphorylated Shc to prevention of abnormal apoptosis. This may involve identifying the participation of other downstream proteins, such as caspases,

activation of pathways previously shown to be influenced by Shc or Ras, such as the Erk/MAPK or PI-3K pathway, or introduction of new players, such as c-Abl. Second, the spatiotemporal mechanisms of Shc signaling in pro-B cells should be examined. For example, activation of the Ras/MAPK pathway is demonstrated to lead to proliferation and survival, and paradoxically, to cell death. Although this is not fully understood, the current paradigm involves subcellular localization as well as the kinetics of activation of these proteins (reviewed in [231]). Finally, the data presented here specifically target the requirement for Shc at stages prior to pre-BCR expression. In order to fully understand both Shc signaling as well as a better illumination of the requirements during B cell development, the role for Shc in later stages of B cell development as well as during the humoral antibody response should be considered. Therefore, additional experimental approaches and systems will lead to a broader understanding of Shc function as well as the larger picture of B cell development.

Appendix I

The c-Abl kinase interacts with phosphorylated Shc

Abstract

Shc is phosphorylated on three critical tyrosine residues that are critical for many of its cellular functions. The adaptor protein Grb2 binds phosphorylated Shc, yet this interaction cannot account for the many signaling pathways in which Shc is thought to function. Here, I describe a modified yeast two-hybrid system I designed to screen for additional proteins that can interact with phosphorylated Shc. Through this approach I identify c-Abl as a potential phospho-Shc binding protein. I use both the full-length c-Abl as well as the c-Abl SH2 domain to show that c-Abl can interact with both overexpressed as well as endogenous Shc protein. Furthermore, interaction of the c-Abl SH2 domain with Shc CH region is dependent upon the phosphorylation of three critical tyrosine residues. Together, these data demonstrate that c-Abl can interact with phosphorylated Shc and provide an interesting and new potential player that can interact with Shc during intracellular signal transduction.

Introduction

The c-Abl tyrosine kinase is a ubiquitously expressed protein that is related to the Src family of kinases. The Abl family of non-receptor tyrosine kinases is composed of c-Abl (*Abl1*) and Arg (*Abl2*). *C-Abl* encodes two c-Abl proteins (type I and type IV) that are encoded from alternate splicing of sequences within the first exon [232]. The type IV form of c-Abl contains a myristoylation consensus sequence and has been shown to be myristoylated *in vivo* [233]. Mutation of the c-Abl myristoylation sequence produces variants that do not associate with the plasma membrane [234]. Additional studies suggest a role for the myristoyl modification in c-Abl autoinhibition by associating with the C-terminal lobe of the catalytic domain [235, 236]. C-Abl can localize to several areas of the cell, including the nucleus, plasma membrane, and actin cytoskeleton [237], and numerous studies have shown that c-Abl catalytic activity is regulated at several levels by different mechanisms.

The N-terminus of c-Abl contains the kinase domain along with Src homology regions 3 and 2 (SH3 and SH2). A short linker connects the SH3 domain to the SH2 domain. The SH2 domain binds peptides containing the Y(p)XXP motif. The SH2 domain is then connected to the tyrosine kinase domain with another linker. The C-terminus of c-Abl contains both a nuclear export signal (NES) [238] and three nuclear localization sequences (NLS) [239, 240], allowing it to shuttle between the nucleus and cytoplasm. In addition, the C-terminus contains a DNA-binding domain [241] and an actin-binding domain that can interact with G- and F-actin [242, 243]. C-Abl has different effects upon the cell depending upon its location. Immunofluorescence of overexpressed c-Abl shows it is primarily present in the nucleus of fibroblasts. However,

the overexpressed transforming c-Abl mutants localize to the cytoplasm and plasma membrane [240].

The tyrosine kinase activity of c-Abl is tightly regulated *in vivo*. This is demonstrated by the inability of c-Abl to transform cells when overexpressed [233]. The N-terminal SH3 domain binds peptides with the PXXP motif and is important for maintaining c-Abl in an inactive state. Deletion [233, 244] or point mutations [245] of the SH3 domain can activate the transforming potential of c-Abl in fibroblasts and hematopoietic cells, although it should be noted that cytoplasmic localization accompanies mutations in c-Abl that activate its transforming potential.

Dysregulation of c-Abl can also occur naturally through chromosome translocations. In humans, the *BCR-ABL* oncogene is a result of reciprocal translocation between chromosome 9 (location of *ABL*) and 22 (location of *BCR*), designated t(9;22) [246]. The cytoplasmic BCR-ABL protein can cause myeloproliferative diseases such as chronic myeloid leukemia (CML) and B-lymphoblastic leukemia (B-ALL). C-Abl can also fuse with a member of the Ets family of transcription factors, *TEL*, located on human chromosome 12. This chromosomal translocation creates another fusion protein that possesses activated c-Abl kinase activity [247, 248]. Bcr-Abl and Tel-Abl are both capable of activating kinases that contribute to cell proliferation and survival, such as Erk/MAPK and Akt/PKB [249].

Nuclear Functions of c-Abl

Overexpression of c-Abl does not lead to cell transformation. Rather, it results in an inhibition of cell growth [239, 250]. When c-Abl is overexpressed, it primarily

localizes to the nucleus in fibroblasts [240] and murine hematopoietic cells [251]. This is allowed by the multiple NLS present in the C-terminus of the protein [239, 240]. Deletion of all NLS abolishes the cytostatic effect of c-Abl overexpression [239]. Rather unexpectedly, overexpression of transforming Abl mutants can have a negative effect on growth [250, 252], although this only occurs when the protein localizes to the nucleus [239]. Inhibition of the transforming BCR-ABL tyrosine kinase activity through mutation or the drug STI571 stimulates its nuclear entry [253]. Trapping BCR-ABL in the nucleus by combining STI571 to stimulate nuclear entry with leptomycin B to block nuclear export effectively killed BCR-ABL transformed cells [253].

The role of nuclear c-Abl is linked to genotoxic stress and DNA-damaging agents [254, 255]. C-Abl is activated in response to multiple inducers of apoptosis, including cisplatin [254, 256], ionizing radiation [257, 258], mitomycin C, etoposide, camptothecin, and TNF [259].

C-Abl can be phosphorylated by cyclin-dependent protein kinase 2 (Cdc2 kinase; its activity is essential for the G1/S transition of the cell cycle) [260], and this inhibits binding of c-Abl to DNA [241]. Furthermore, phosphorylation of c-Abl has been shown to occur during mitosis, causing a loss of DNA-binding activity [241]. This suggests a function for c-Abl in the cell cycle.

The tyrosine kinase activity of c-Abl can be regulated during the cell cycle by interaction with the retinoblastoma protein (Rb). RB was identified as a tumor suppressor protein that functioned as a regulator of cell cycle progression at the G1 checkpoint, but its cellular functions have expanded since its discovery to include roles in cellular differentiation and regulation of apoptosis (reviewed in [261]). Rb can bind the

ATP-binding lobe of c-Abl with a C-terminal protein-binding domain, resulting in inactivation of the c-Abl kinase during G0/G1 [262]. Hyperphosphorylation of Rb releases this inhibitory interaction, allowing c-Abl to become active in the S-phase of the cell cycle. Interestingly, *Rb*-deficient cells are more sensitive to DNA damage-induced cell death [263]. During apoptosis, Rb is actively cleaved by caspases and degraded [264, 265]. This is also observed when cells are subjected to inducers of genotoxic stress [264]. Degradation of Rb under these apoptosis-inducing conditions may release the inhibitory effect on c-Abl, allowing it to become active. Consistent with this idea, addition of caspase inhibitors can block the activation of c-Abl in response to etoposide treatment [259].

Recently c-Abl has been shown to interact with proliferating cell nuclear antigen (PCNA). PCNA belongs to the family of DNA sliding clamps, is a cofactor of DNA polymerase, and is an essential component for DNA replication (reviewed in [266]). C-Abl interacts with PCNA through a PIP (PCNA-Interacting Protein) region in the SH2 domain, allowing PCNA to ubiquitylate c-Abl for proteosome-mediated degradation [267]. SiRNA-mediated knockdown of c-Abl or inhibition of c-Abl activity by Gleevec reduced apoptosis in MCF7 cells treated with cisplatin to induce DNA damage. Furthermore, when c-Abl expression was restored, apoptosis in response to cisplatin was regained, but this was dependent upon the presence of PCNA and the PIP box of c-Abl [267].

Cytoplasmic function of c-Abl

The cytoplasmic function of c-Abl is less understood than its nuclear role. Activation of nuclear c-Abl is associated with apoptosis, yet mice deficient in *c-Abl* show high post natal mortality rate and severe T and B cell lymphopenia [227, 228]. In addition, bone marrow transfers of mice with $Abl^{m1/m1}$ donors (Abl^{m1} mice have germline deletion of the C-terminal third of c-Abl) are severely impaired in their ability to regenerate B cell precursors when compared to littermate controls, demonstrating that the B lymphopenia in *c-Abl*-deficient mice is cell-intrinsic [227]. Since loss of *c-Abl* leads to death of early B lymphocytes, it appears that c-Abl also plays a role in survival.

Many studies have focused on the signaling pathways surrounding transforming c-Abl fusion proteins, such as BCR-ABL, TEL-ABL, and v-Abl. These Abl proteins, as well as transforming c-Abl mutants, are localized to the cytoplasm. This suggests a mitogenic role for cytoplasmic Abl, as opposed to its nuclear role. Although these studies may not have direct application to the requirements of endogenous c-Abl:phospho-Shc interactions, they clearly demonstrate that oncogenic Abl mutants and Shc are functionally linked. ShcY239F/Y313F has diminished capacity to bind v-Abl, and expression of this mutant Shc decreases the transforming ability of pre-B cells by v-Abl [162]. This loss of transforming ability was associated with decreased Ras, Raf, and Erk activation. In addition, BCR-ABL can protect Ba/F3 cells from apoptosis due to cytokine withdrawal, and loss of BCR-ABL in K562 cells sensitizes these cells to apoptotic stimuli [234, 268].

Results

Identification of proteins that interact with phosphorylated Shc

To identify proteins that interact with phosphorylated Shc, I employed the yeast two-hybrid system. This system is useful to identify novel protein interactions. However, proteins in this system are not phosphorylated. To address this caveat, I fused the kinase domain of human LCK (encoding amino acids 226 – 509) to the C-terminus of the Shc CH region (human SHC, encoding amino acids 208-377). I chose the LCK kinase because it is demonstrated to efficiently phosphorylate Shc *in vivo* [269]. I used only the CH1 region of Shc to avoid detecting proteins that interacted with the PTB or SH2 domains of Shc. The ShcCH-LCK sequence was then inserted into the yeast pGBT9 vector, which fuses the Gal4 DNA binding domain (DBD) to the N-terminus of ShcCH-LCK (Figure A1-1). In the yeast system, the Gal4 DBD fused to ShcCH-LCK will interact with the Gal4 promoter in the nucleus. Prey proteins possess the Gal4 activation domain. If a prey protein can interact with phosphorylated ShcCH-LCK, the Gal4 activation domain will be brought into proximity to allow transcription of His (thereby rescuing the His auxotrophy), and yeast will be permitted to grow on selective medium (Figure A1-1). As a negative control, I created ShcCH^{FFF}-LCK. This allows the negative selection of proteins that bind in a phoshotyrosine-independent manner to ShcCH or the LCK kinase domain. I also created ShcCH^{FFY}-LCK and ShcCH^{YYF}-LCK to determine if Shc binding proteins bound specifically to pY239/pY240 or to pY317. As a proof-of-principle experiment, I also cloned the SH2 domain of Grb2 into the pVP16 prey vector. This was used as a positive control for a protein that is known to interact specifically with phosphorylated Shc.

ShcCH-LCK is phosphorylated in yeast

To determine if the LCK kinase domain could phosphorylate Shc, I transformed HF7c yeast with various ShcCH-LCK constructs or ShcCH (no LCK kinase domain). Total cell lysates were probed with antibodies specific for either pY230/pY240 or pY317 of Shc (Figure A1-2). As I hypothesized, the ShcCH-LCK fusion proteins were phosphorylated on tyrosine resides and detected with Shc phosphorylation-specific antibodies. No phosphorylation was observed when the tyrosine residues were mutated to phenylalanine. In addition, the ShcCH region was not phosphorylated in the absence of the LCK kinase domain. This suggested that the ShcCH-LCK fusion protein created a docking site for potential proteins that bind phosphorylated Shc. Moreover, The $ShcCH^{FFF}$-LCK and ShcCH proteins provided excellent negative controls as ShcCH regions that were not phosphorylated.

Grb2SH2 interacts specifically with ShcCH-LCK in yeast

As a proof-of-principle experiment, I tested whether the ShcCH-LCK fusion protein could interact with the SH2 domain of Grb2. The Grb2 SH2 domain is known to bind to phosphorylated Shc, and this interaction is abolished if the three critical tyrosine residues are mutated to phenylalanine. Yeast transformed individually with Grb2SH2, ShcCH-LCK, or $ShcCH^{FFF}$-LCK all grew on non-selective medium, demonstrating that these proteins were not toxic to the yeast (Figure A1-3). Yeast transformed with these construct were unable to efficiently grow on selective medium, demonstrating that these proteins could not individually rescue all the auxotrophies of HF7c yeast. However, cotransformation of yeast with ShcCH-LCK and Grb2SH2 permitted growth on selective

medium. This suggests that the Grb2 SH2 domain is able to interact with the phosphorylated tyrosine residues within the Shc CH region. Furthermore, growth was not permitted on selective medium if these tyrosine residues were not present, as demonstrated with yeast cotransformed with ShcCH^{FFF}-LCK and Grb2SH2. This suggests that the ShcCH-LCK fusion protein permits interaction of physiologically relevant target proteins with the CH region of Shc in yeast specifically when this region is phosphorylated on the three critical tyrosine residues.

Figure A1-1. **Schematic of modified yeast two-hybrid**. Fusion of the Lck kinase domain to the ShcCH region allows Lck kinase to phosphorylate the tyrosine residues Y239, Y240, and Y317 within the ShcCH region. Fusion of the Gal4 DNA binding domain (DBD) to the ShcCH region allows this protein to interact with the Gal4 promoter. Prey proteins, such as the SH2 domain of Grb2, can interact with the phosphorylated residues on ShcCH. This brings the Gal4 activation domain into proximity to activate transcription of His. This rescues the His auxotrophy and permits growth on selective medium.

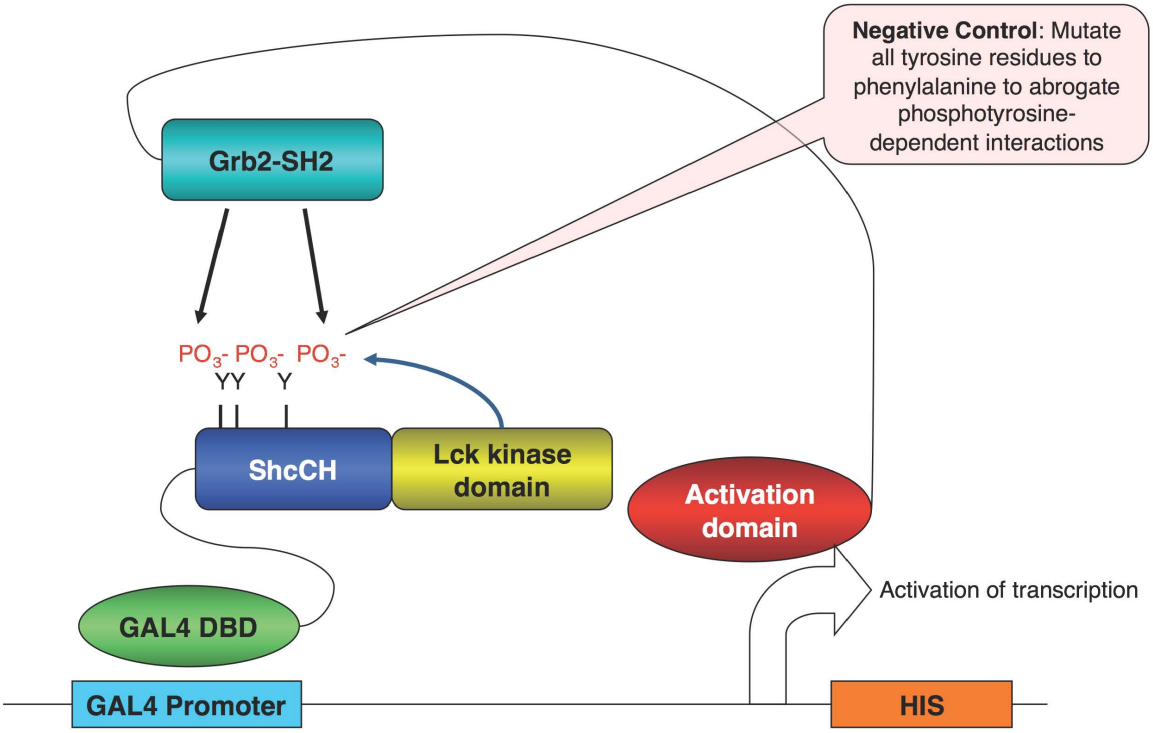

Negative Control: Mutate all tyrosine residues to phenylalanine to abrogate phosphotyrosine-dependent interactions
Grb2-SH2
PO3- PO3- PO3-
YY Y
ShcCH
Lck kinase domain
Activation domain
Activation of transcription
GAL4 DBD
GAL4 Promoter
HIS

Figure A1-2. **ShcCH-LCK fusion proteins are phosphorylated on tyrosine residues in yeast**. HF7c yeast were transformed with the indicated ShcCH-LCK variant (or ShcCH without LCK fusion). Cells were lysed, and total cell lysates were examined with phospho-specific antibodies to phosphorylated Shc Y239/Y240 (left panel) or to phosphorylated Shc Y317 (right panel).

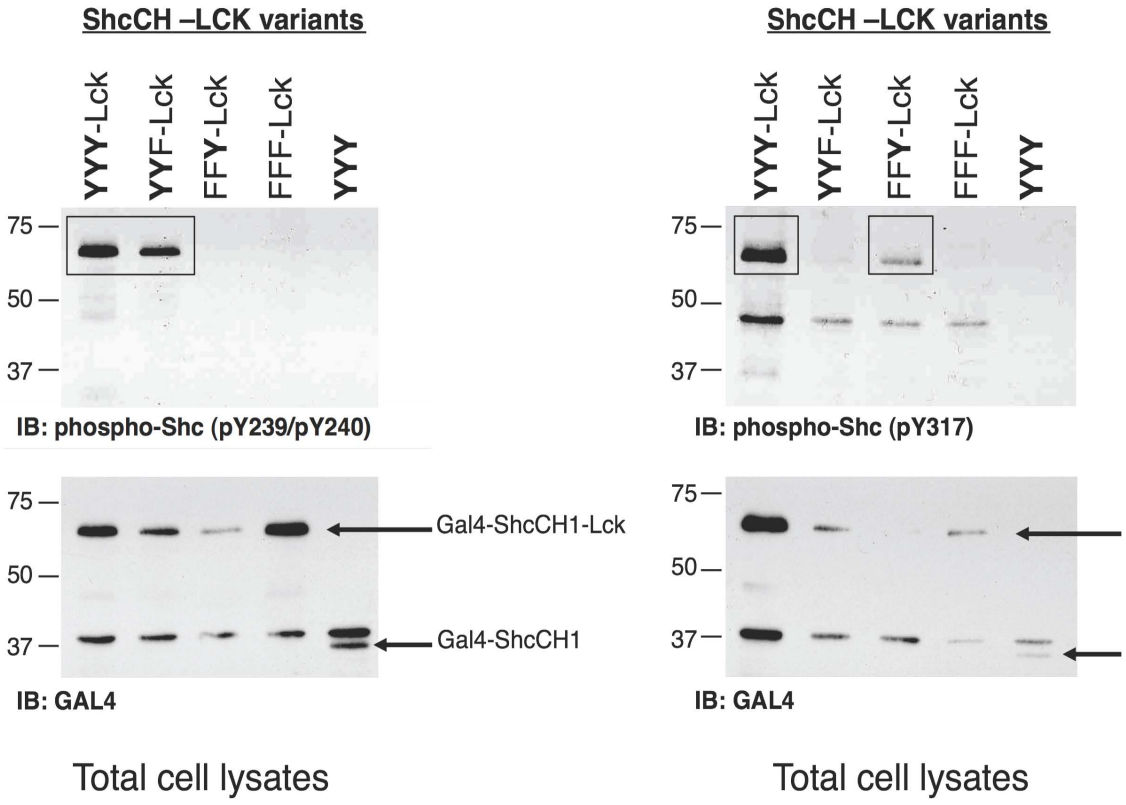
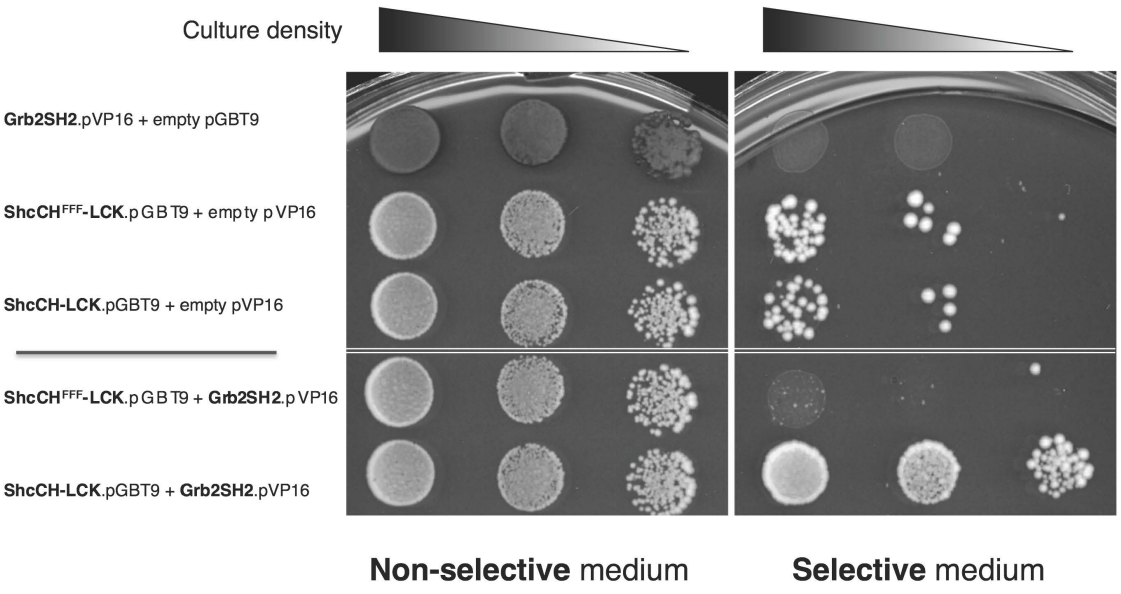

ShcCH –LCK variants
YYY-Lck
YYF-Lck
FFY-Lck
FFF-Lck
YYY
75
50
37
IB: phospho-Shc (pY239/pY240)
Gal4-ShcCH1-Lck
Gal4-ShcCH1
IB: GAL4
Total cell lysates
ShcCH –LCK variants
YYY-Lck
YYF-Lck
FFY-Lck
FFF-Lck
YYY
75
50
37
IB: phospho-Shc (pY317)
IB: GAL4
Total cell lysates

Figure A1-3. **The SH2 domain of Grb2 interacts specifically with phosphorylated ShcCH in yeast**. HF7c yeast were transformed with the indicated constructs. The top three rows show yeast transformed with the single construct; the bottom two rows show yeast transformed with the indicated construct as well as with Grb2SH2.pVP16 to detect protein interactions. After transformation, yeast were plated on SD-LW plates (non-selective; only selects for presence of plasmid) or SD-LWH with 10 mM 3-AT (selective; requires interaction of bait and prey proteins for growth).


Culture density
Grb2SH2.pVP16 + empty pGBT9
ShcCHFFF-LCK.pGBT9 + empty pVP16
ShcCH-LCK.pGBT9 + empty pVP16
ShcCHFFF-LCK.pGBT9 + Grb2SH2.pVP16
ShcCH-LCK.pGBT9 + Grb2SH2.pVP16
Non-selective medium
Selective medium

Identification of new proteins that bind phosphorylated Shc

After validating the ability of ShcCH-LCK to interact with proteins that bind specifically to the phosphorylated residues within the ShcCH region, I used the ShcCH-LCK construct to probe a seven-day mouse embryo library. Plasmid DNA from colonies that grew on selective medium was further screened to remove candidates that were not absolutely dependent on the phosphorylation of tyrosine residues in the CH1 region for growth in the yeast two-hybrid assay. Candidate plasmid DNA were transformed into HF7c yeast with ShcCH (no LCK), ShcCH^{FFF}-LCK, empty pGBT9, or once again into yeast with ShcCH-LCK. Only plasmids that permitted growth with ShcCH-LCK but not with ShcCH, ShcCH^{FFF}-LCK, or empty pGBT9 were sequenced. The results of the sequenced proteins are listed in Table A1-1. Interestingly, all sequences identified contained an SH2 domain, which mediates binding to phosphorylated tyrosine residues. Of the proteins identified, I chose to study c-Abl and its interaction with phosphorylated Shc.

The SH2 domain of c-Abl binds phosphorylated Shc

I first sought to determine if the ShcCH-LCK fusion protein could interact with the c-Abl SH2 domain in a mammalian system. To achieve this, I inserted the ShcCH-LCK and c-Abl SH2 sequences into mammalian expression vectors, tagging the ShcCH-LCK constructs to GST and the c-Abl constructs to a triple HA sequence. I co-transfected 293T cells with ShcCH-LCK or ShcCH^{FFF}-LCK and FL c-Abl or c-Abl SH2. Then I immunoprecipitated lysates with anti-HA to pull out the c-Abl proteins and looked for association of ShcCH-LCK proteins. Importantly, the ShcCH-LCK was

phosphorylated as demonstrated with phosphorylation-specific Shc antibodies whereas ShcCH^{FFF}-LCK was not (Figure A1-4). The c-Abl SH2 protein selectively co-immunoprecipitated ShcCH-LCK but not ShcCH^{FFF}-LCK, demonstrating the requirement for the phosphorylated tyrosine residues of Shc to interact with the c-Abl SH2 domain. Rather unexpectedly, the full-length c-Abl protein was able to co-immunoprecipitate ShcCH-LCK independently of its phosphorylation status. It is not clear whether the full-length c-Abl is interacting with the CH region of Shc or the LCK kinase domain. The full-length c-Abl protein contains an SH3 domain, which could mediate binding to PXXP motifs within the CH region of Shc.

I wanted to move my analyses closer to detection of an endogenous interaction. Since Shc is highly expressed and phosphorylated in 293T cells, I transfected these cells with either empty vector or the c-Abl SH2 domain. The Abl SH2 domain was able to co-immunoprecipitate endogenous Shc from 293T cells (Figure A1-5). This experiment was repeated to include the full-length c-Abl, and the full-length c-Abl as well as the c-Abl SH2 domain were both able to co-immunoprecipitate endogenous Shc from 293T cells (Figure A1-6). Although, as mentioned from Figure A1-5, it is not clear whether the full-length c-Abl interacted with Shc in a phosphotyrosine-dependent manner.

Table A1-1. **Proteins identified from yeast two-hybrid that interact specifically with ShcCH-LCK.** HF7c yeast containing ShcCH-LCK were transformed with a seven-day mouse embryo library. I screened three million colonies and obtained 187 candidates. Plasmid DNA from 89 of these candidates was further transformed into HF7c yeast containing ShcCH (without LCK), ShcCH^{FFF}-LCK, or empty pGBT9 vector. Only the candidates that permitted growth with ShcCH-LCK (but not with any of the other constructs) were sequenced.

Clone	GI Number	# of Clones Identified	Predicted Domains
Grb2	34328126	5	SH2
Grb7	51858458	1	SH2
Grb10	70909310	6	SH2
Abl-1	33859503	3	SH3, SH2
Abl-2	68139001	3	SH3, SH2
Fyn	74140049	3	SH3, SH2
Src	76253930	1	SH2
PLCγ1	40675438	3	SH2, SH2
Srms	54287681	1	SH3, SH2
PI3Krα	68299808	2	SH2
PI3Krβ	55250649	3	SH2

Figure A1-4. **ShcCH-LCK (but not ShcCH^{FFF}-LCK) co-IPs with FL c-Abl and Abl SH2.** 293T cells were transiently transfected with GST-tagged ShcCH-LCK or ShcCH^{FFF}-LCK and HA-tagged full-length c-Abl or the c-Abl SH2 domain that was cloned from a positive yeast two-hybrid colony. Cells lysates were immunoprecipitated with anti-HA agarose beads. Immunoprecipitates and total cell lysates were immunoblotted with anti-GST to detect ShcCH-Lck proteins, anti-phospho-Shc (pY239/pY240) to detect phosphorylation of the ShcCH-Lck proteins, and anti-HA to detect c-Abl constructs.

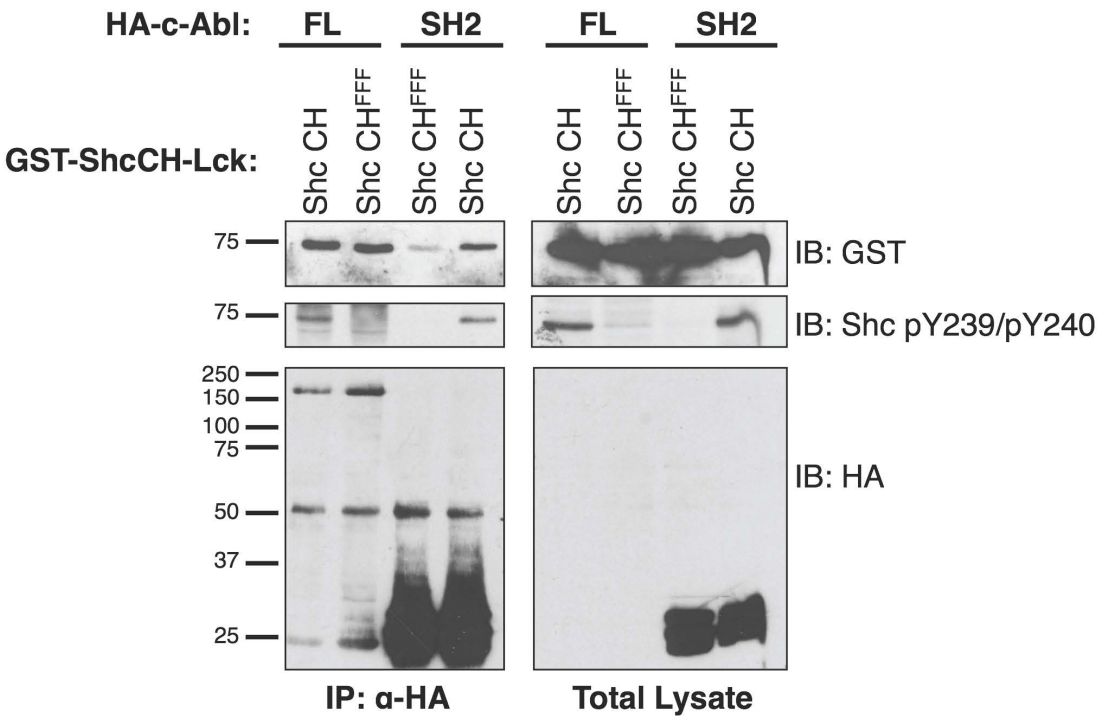

HA-c-Abl:
FL
SH2
FL
SH2
GST-ShcCH-Lck:
Shc CH
Shc CH^FFF
Shc CH^FFF
Shc CH
Shc CH
Shc CH^FFF
Shc CH^FFF
Shc CH
75
75
250
150
100
75
50
37
25
IB: GST
IB: Shc pY239/pY240
IB: HA
IP: α-HA
Total Lysate

Figure A1-5. **Abl SH2 can co-IP endogenous Shc.** 293T cells were transiently transfected with HA-tagged c-AblSH2. Cells lysates were immunoprecipitated with anti-HA agarose beads. Immunoprecipitates and total cell lysates were immunoblotted with anti-HA to detect c-Abl SH2 and anti-Shc to detect endogenous Shc protein.

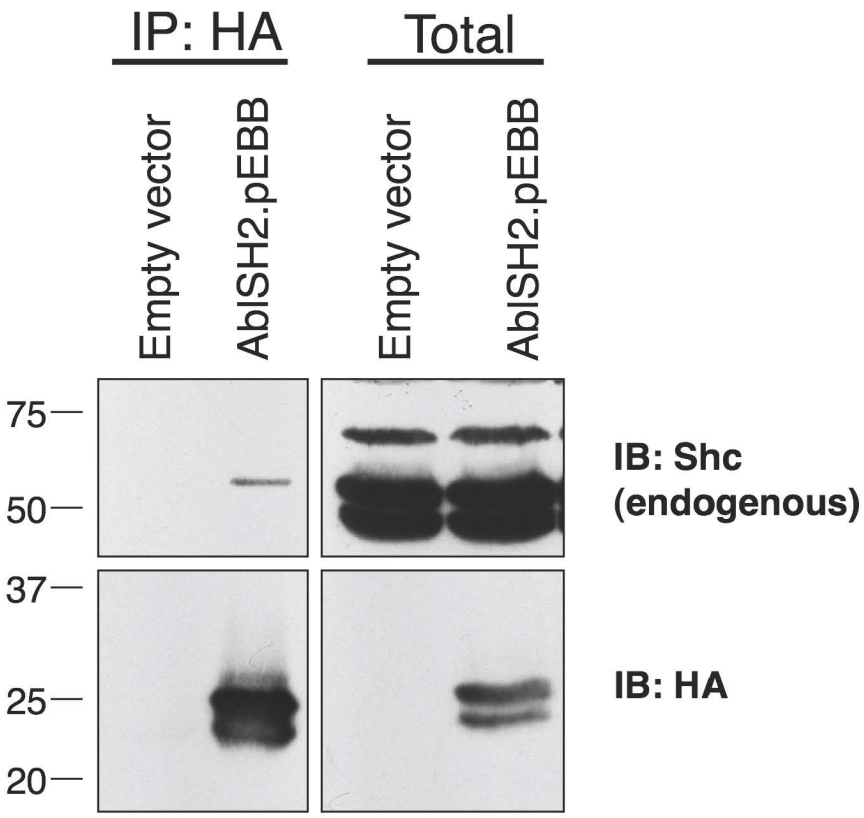
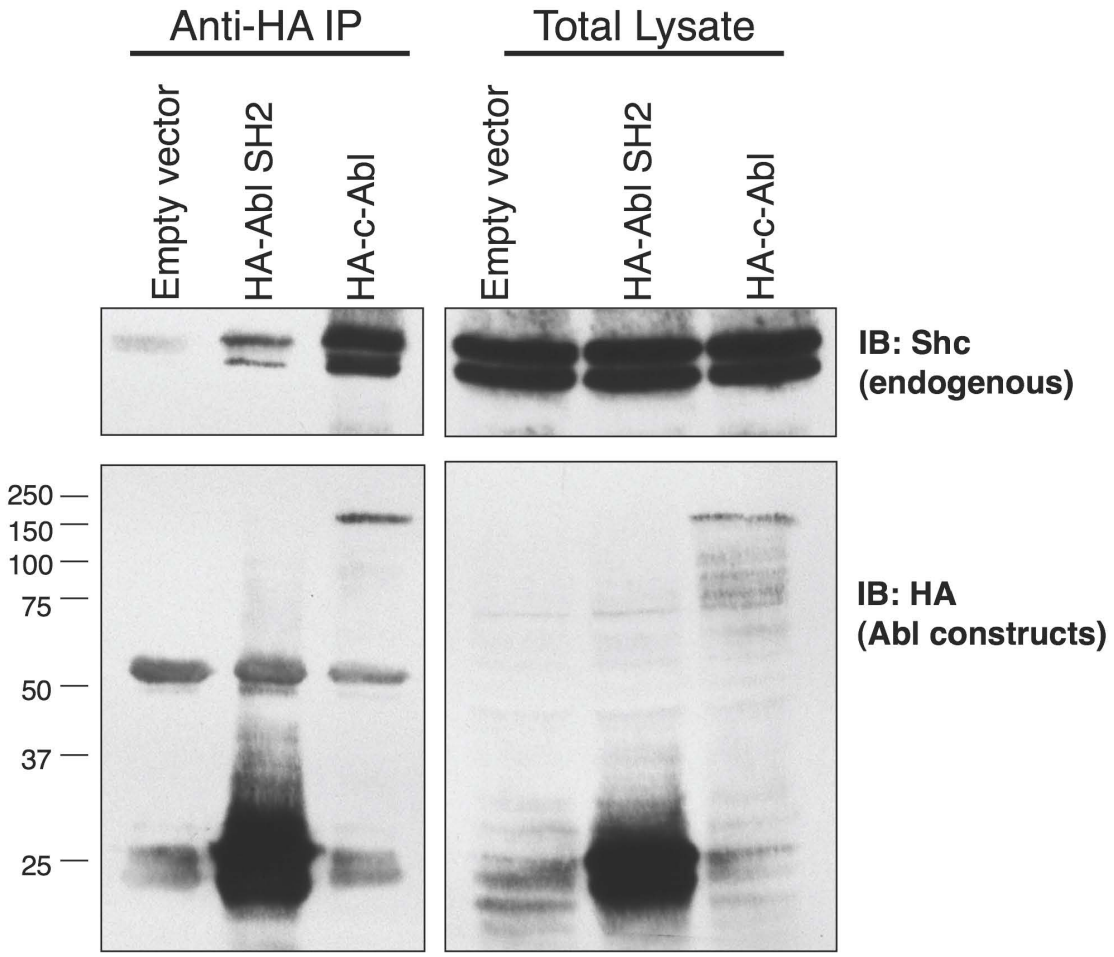

IP: HA
Total
Empty vector
AblSH2.pEBB
Empty vector
AblSH2.pEBB
75
50
37
25
20
IB: Shc
(endogenous)
IB: HA

Figure A1-6. **Abl SH2 and FL c-Abl can co-IP endogenous Shc**. 293T cells were transiently transfected with HA-tagged c-AblSH2 of full-length c-Abl. Cells lysates were immunoprecipitated with anti-HA agarose beads. Immunoprecipitates and total cell lysates were immunoblotted with anti-Shc to detect endogenous Shc protein and anti-HA to detect c-Abl constructs.


Anti-HA IP
Total Lysate
Empty vector
HA-Abl SH2
HA-c-Abl
Empty vector
HA-Abl SH2
HA-c-Abl
IB: Shc
(endogenous)
250
150
100
75
50
37
25
IB: HA
(Abl constructs)

Discussion

In this chapter I have created a modified yeast two-hybrid assay with which to detect proteins that interact with phosphorylated Shc. By identifying new proteins that bind phosphorylated Shc, I hoped to further illuminate the signaling pathways associated with Shc phosphorylation *in vivo*. I used only the CH region of Shc as the bait for the yeast two-hybrid and fused it to the kinase domain of LCK to permit a continuous source of phosphorylation. I also created two important negative controls: the ShcCH^{FFF}-LCK and the ShcCH (lacking LCK). The ShcCH^{FFF}-LCK protein is identical to the ShcCH-LCK with the exception that it lacks the three critical tyrosine residues and therefore cannot become phosphorylated on these residues. The ShcCH protein is identical to ShcCH-LCK except that the former lacks the LCK kinase domain to phosphorylate tyrosine residues within the CH1 region. By using these three proteins, I effectively eliminated detection of prey proteins produced from the seven-day mouse embryo library that might bind the Shc PTB or SH2 domain and created a means to screen against proteins that bound to LCK kinase or the ShcCH-LCK fusion protein in a phosphotyrosine-independent manner. Due to the high concentration of proline residues in the Shc CH1 region and the SH3 domains produced from the seven-day mouse embryo library, it was critical to screen against these non-phosphotyrosine-dependent interactions. This resulted in a highly selective yeast two-hybrid screening that identified 10 protein candidates that interacted with phosphorylated Shc. These candidates included, but were not limited to, Grb2.

I chose to examine the association of c-Abl with phosphorylated Shc due to multiple reasons. First, c-Abl and Shc are both expressed in lymphocytes. This permits

their potential interaction in B lymphocytes. In addition, Shc and c-Abl are both linked to cellular transformation, suggesting that both proteins contribute toward oncogenic potential. Expression of c-Abl is similar among B cell subsets, yet pro-B cells show the greatest level of c-Abl phosphorylation, suggesting it is active during the pro-B stage of development [226]. Finally, disruption of Shc signaling with conditional expression of ShcFFF blocks early B cell development, and mice with disruption of *c-Abl* show similar developmental defects, including reduction in pro-B and pre-B populations, elevated apoptosis without compromised cell cycle progression during *ex vivo* culture, and increased apoptosis during IL-7 withdrawal [229, 230].

The SH2 domain is required for the transforming ability of activated c-Abl [270]. Quite interestingly, the SH2 domains of Src and the p85 subunit of PI-3K were previously demonstrated to provide a competent substitution for the SH2 domain of c-Abl [271]. The c-Abl, Src, and p85 subunit of PI-3K were all identified in the yeast two-hybrid analysis for proteins that bind phosphorylated Shc. The SH3 domain of c-Abl is required for regulation of kinase activity, and the SH2 domain is required for transforming ability. It is interesting to speculate that the SH2 domain of c-Abl may interact with phosphorylated Shc in the cytoplasm, thereby contributing to its transforming abilities [271].

I feel there are two potential mechanisms by which Shc could affect c-Abl-mediated cellular events. First, phosphorylated Shc may act as a docking site to maintain c-Abl in the cytoplasm during signal transduction events. This could prevent the accumulation of c-Abl in the nucleus that could lead to apoptosis. Second, interaction of phosphorylation of Shc with c-Abl may contribute to a proliferative function of c-Abl

in the cytoplasm by activating pathways such as the Ras/MAPK pathway. These two potential outcomes of phospho-Shc:c-Abl interaction are not mutually exclusive, and it is quite possible that one could potentiate the other.

The potential for Shc phosphorylation to increase cytoplasmic accumulation of c-Abl could be quickly tested in cell lines. The Ba/F3 pro-B cell line could be transiently transfected with wild-type Shc or ShcFFF, and the comparison of cytoplasmic versus nuclear Abl compared with Western blotting. If detection of endogenous c-Abl proves difficult (as I have previously found), a tagged c-Abl construct could be utilized to assist in detection of c-Abl. In addition, the cytoplasmic versus nuclear localization of c-Abl in primary mouse pro-B cells of *Mb1*-Cre versus *Mb1*-Cre/ShcFFF could be examined.

The ability of the phosho-Shc:c-Abl interaction to prevent apoptosis of pro-B cells could be tested by activating c-Abl independently of Shc. Multiple cytoplasmic localized c-Abl mutants exist. Furthermore, bone marrow is easily infected with retroviral constructs. It would be interesting to test the ability of ShcFFF-expressing pro-B cells to respond to IL-7 after infection with various activated c-Abl mutants. In addition, the phosphorylation status of c-Abl in ShcFFF-expressing pro-B cells should be examined to determine if ShcFFF impairs c-Abl activation.

Other aspects of pro-B cell development in *Mb1*-Cre/ShcFFF mice could be examined to check for c-Abl function. Pro-B cells from *c-Abl*-deficient mice show markedly reduced V(D)J rearrangement but normal levels of signal-end intermediates, suggesting that c-Abl may participate in joining the coding ends during V(D)J rearrangement [229]. Although this data would only add correlative evidence for the

c-Abl:phospho-Shc interaction, it may provide additional insight into the mechanism behind pro-B cell loss in ShcFFF-expressing pro-B cells.

Unfortunately, the phosphotyrosine-independent interaction of Shc with c-Abl will likely complicate the understanding of the phospho-Shc:c-Abl interaction. However, by identifying proteins that are capable of binding phosphorylated Shc, I feel this work has aided to further the understanding of potential protein:protein interactions that can occur during signaling events that contribute toward receptor signal transduction via Shc.

Appendix II

Cell migration, peripheral B stimulation, and attempted rescue of the *Mb1*-Cre/ShcFFF phenotype

In this appendix, I have included several pieces of experimental data that do not easily fit into other sections of this dissertation. Although some data require repeating before solid conclusions can be made, I felt it was important to include these data for any future scientists who may consider some questions that I have already lightly tested.

Results/Discussion

Mb1-Cre/ShcFFF mice express normal levels of Bcl-2 transcript, and B lymphopoiesis is not rescued with transgenic expression of Bcl-2.

After identifying the abnormal apoptosis in *Mb1*-Cre/ShcFFF early B cells, I sought to determine if the levels of *Bcl2* transcript could be depressed due to the presence of ShcFFF. I performed real-time PCR on freshly isolate bone marrow B cell subsets but did not notice any decrease in the levels of *Bcl2* RNA between control and ShcFFF-expressing B subsets (Figure A2-1). This suggests that ShcFFF does not depress *Bcl2* transcript levels during B cell development.

The real-time PCR data was supported in *Mb1*-Cre/ShcFFF mice transgenic for Bcl2. These mice constitutively express Bcl2 in both the B and T lineages. Although I was only able to analyze one set of mice, it is quite apparent that the presence of Bcl2 was not capable of rescuing the decreased B cell numbers resulting from ShcFFF (Figure A2-2). Although additional data is necessary to make any firm conclusions, Bcl2 appears

to be unable to rescue the defect due to ShcFFF, and ShcFFF does not appear to depress endogenous production of *Bcl2* RNA. Since the Bcl-2-regulated apoptosis pathway relies upon a balance of multiple pro- and anti- apoptotic proteins, it is necessary to examine other Bcl-2 family transcript levels to determine their potential influence in the heightened apoptosis in *Mb1*-Cre/ShcFFF mice.

Figure A2-1. ***Mb1*-Cre/ShcFFF mice are not defective in *Bcl2* transcripts**. Freshly isolated bone marrow from *Mb1*-Cre or *Mb1*-Cre/ShcFFF littermate mice was FACS sorted into the pro-B, pre-B, immature, and mature B cell subsets. RNA from these subsets was collected and used to create cDNA. Real-time PCR was performed to examine the levels of *Bcl2*. Graph depicts the percentage of *Bcl2* mRNA levels (normalized to GAPDH) relative to the *Mb1*-Cre pre-B population.

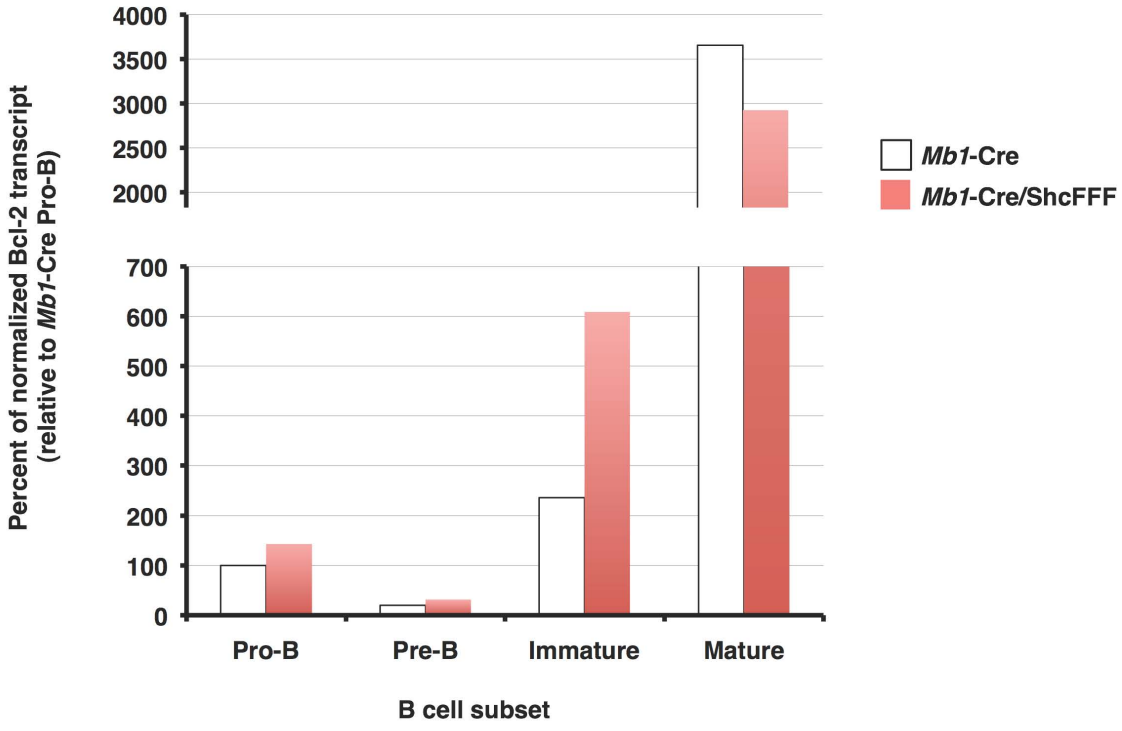
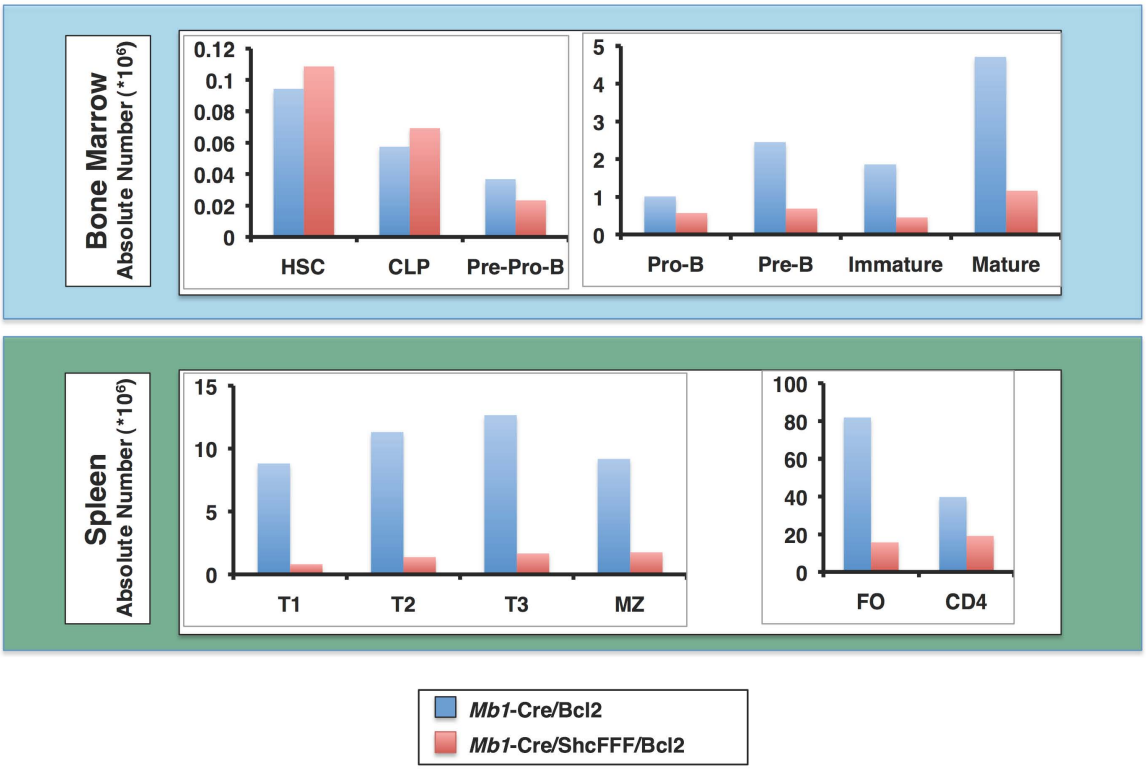

4000
3500
3000
2500
2000
700
600
500
400
300
200
100
0
Percent of normalized Bcl-2 transcript
(relative to Mb1-Cre Pro-B)
Pro-B
Pre-B
Immature
Mature
B cell subset
Mb1-Cre
Mb1-Cre/ShcFFF

Figure A2-2. **Expression of a Bcl2 transgene does not rescue *Mb1*-Cre/ShcFFF B development**. Absolute cell numbers were determined from FACS analysis to identify the indicated lymphocyte subset and total bone marrow counts or spleen from the indicated mouse genotype. Total cell numbers for subsets in the bone marrow numbers (top panel) and spleen (bottom panel) are depicted.


Bone Marrow
Absolute Number (*10⁶)
0.12
0.1
0.08
0.06
0.04
0.02
0
HSC
CLP
Pre-Pro-B
5
4
3
2
1
0
Pro-B
Pre-B
Immature
Mature
Spleen
Absolute Number (*10⁶)
15
10
5
0
T1
T2
T3
MZ
100
80
60
40
20
0
FO
CD4
Mb1-Cre/Bcl2
Mb1-Cre/ShcFFF/Bcl2

Mb1-Cre/ShcFFF bone marrow B cell migrate to SDF-1α

Shc plays a role in signaling downstream of the CXCR4 cytokine receptor. The CXCR4 receptor binds SDF-1α, a stromal-derived cytokine that is produced by the reticular stromal cells within the bone marrow and is demonstrated to be important during B cell development [272-274]. The CXCR4 receptor is expressed on early B cells and promotes migration within the bone marrow microenvironment. I utilized a transwell assay to determine if ShcFFF-expressing bone marrow B cells could migrate toward SDF-1α. I did not observe a significant difference in the percentage of bone marrow cells that migrated to SDF-1α relative to the input or any difference in the fold migration to SDF-1α over BSA in ShcFFF-expressing bone marrow (Figure A2-3). I did not observe a defect in ShcFFF-expressing B cells to migrate to SDF-1α when comparing total bone marrow (assayed with Cell Titer Glo, Figure A2-3) or only the $CD19^{+}$ cell numbers (cell counts and FACS analysis, Figure A2-4). These data, combined with the defective response of ShcFFF-expressing pro-B cells on OP-9 stromal cells, suggest that migration to SDF-1α is not responsible for the defect in ShcFFF-expressing pro-B cells.

Figure A2-3. **ShcFFF-expressing bone marrow migrates normally to SDF-1α**. A transwell migration assay was used to determine the capacity of bone marrow cells from *Mb1*-Cre/ShcFFF mice to migrate to varying concentrations of SDF-1α. Total bone marrow from the indicated mouse was placed into the upper well of a transwell plate, and migration medium containing varying concentrations of SDF-1α was placed in the lower well. The assay was allowed to proceed for 2 hours in a 37°C incubator. After this time, the cells that migrated to the lower chamber as well as a sample from the original input suspension were quantified with Cell Titer Glo. ***A.*** The percent of cells that migrated to the lower chamber relative the the original input. ***B.*** The fold change of cells that migrated to SDF1-α versus BSA.

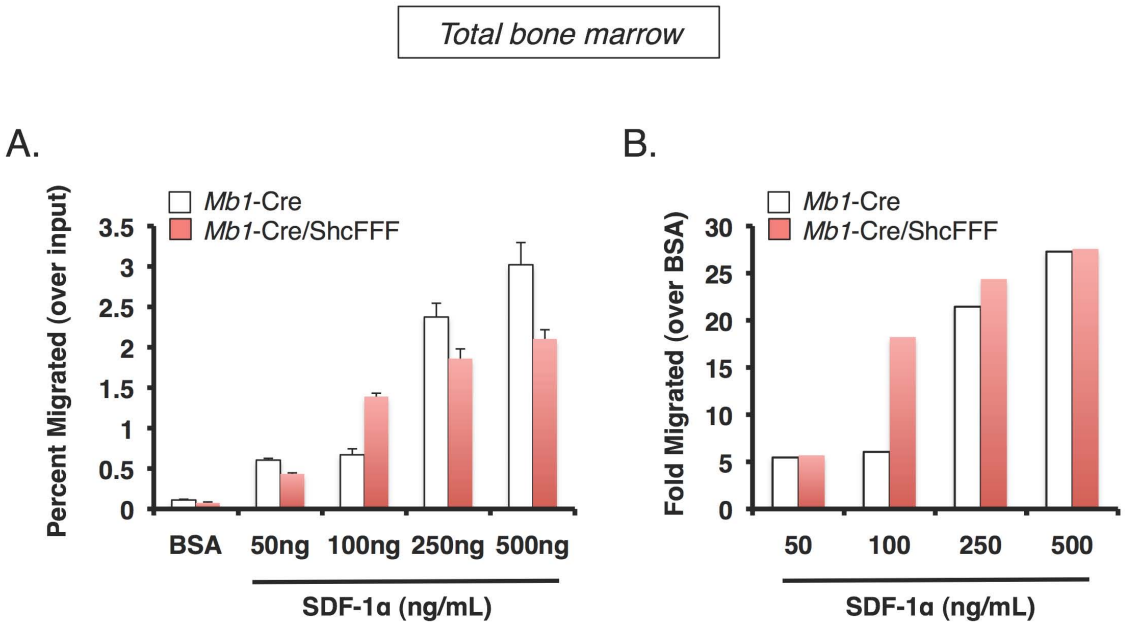
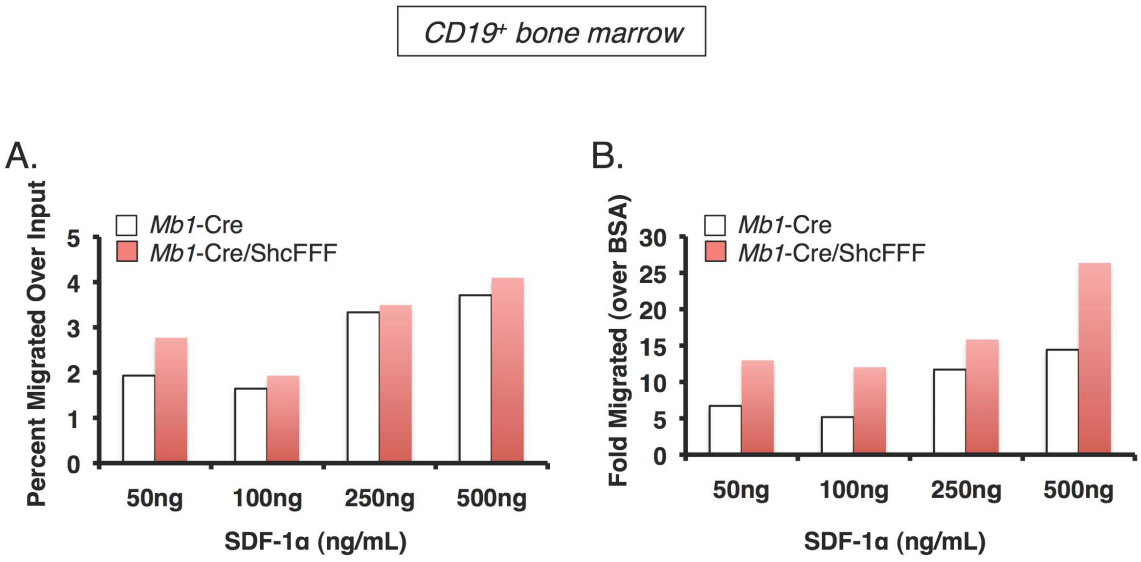

Total bone marrow
A.
Mb1-Cre
Mb1-Cre/ShcFFF
Percent Migrated (over input)
3.5
3
2.5
2
1.5
1
0.5
0
BSA
50ng
100ng
250ng
500ng
SDF-1α (ng/mL)
B.
Mb1-Cre
Mb1-Cre/ShcFFF
Fold Migrated (over BSA)
30
25
20
15
10
5
0
50
100
250
500
SDF-1α (ng/mL)

Figure A2-4. **ShcFFF-expressing CD19$^+$ bone marrow migrates normally to SDF-1α**. A transwell migration assay was used to determine the capacity of bone marrow cells from *Mb1*-Cre/ShcFFF mice to migrate to varying concentrations of SDF-1α as in Figure A2-5. After allowing cells to migrate, the cells that migrated to the lower chamber as well as a sample from the original input suspension were counted by trypan exclusion. FACS analysis was then performed on the migrated and input samples to determine the percentage of CD19$^+$ cells. This analysis, combined with the total cell number, determined the number of CD19$^+$ cells that migrated in this assay. ***A.*** The percent of CD19$^+$ cells that migrated to the lower chamber relative the the original input. ***B.*** The fold change of CD19$^+$ cells that migrated to SDF1-α versus BSA.


CD19+ bone marrow
A.
Percent Migrated Over Input
Mb1-Cre
Mb1-Cre/ShcFFF
0
1
2
3
4
5
50ng
100ng
250ng
500ng
SDF-1α (ng/mL)
B.
Fold Migrated (over BSA)
Mb1-Cre
Mb1-Cre/ShcFFF
0
5
10
15
20
25
30
50ng
100ng
250ng
500ng
SDF-1α (ng/mL)

B cell development is impaired in both young and aged Mb1-Cre/ShcFFF mice

Aging has direct consequences on B cell development. The bone marrow of younger mice contains B cells of fetal origin, which possess a few significant differences from bone marrow-derived B cells. This includes a decreased dependency upon IL-7. Aging results in decreased bone marrow production and slowed cycling of progenitor B cells. However, mature, recirculating B cells are long-lived and accumulate in older mice. I examined mice ranging from four weeks to nine months of age. The difference in pro-B cell numbers was not significant in four-week-old mice. This could be due to the presence of a population of fetal origin progenitors (Figure A2-5, top panel). The pro-B cell defect is maintained into older mice as well as decreased pre-B and immature B cells in *Mb1*-Cre/ShcFFF mice (Figure A2-5, bottom panel). Furthermore, the splenic B cell populations of *Mb1*-Cre/ShcFFF mice were also not rescued with age (Figure A2-6). This is similar to the IL-7-deficient mice in which a decrease in B cell numbers is observed in the spleen, and this defect is not rescued with age.

Figure A2-5. **Effects of aging in *Mb1*-Cre/ShcFFF bone marrow B cells**. Absolute cell numbers were determined from FACS analysis to identify the indicated lymphocyte subset and total bone marrow counts of *Mb1*-Cre/ShcFFF mice versus littermate controls in (top panel) 4-week-old mice, (middle panel) 8-12-week-old mice, and (lower panel) 9-month-old mice. *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$

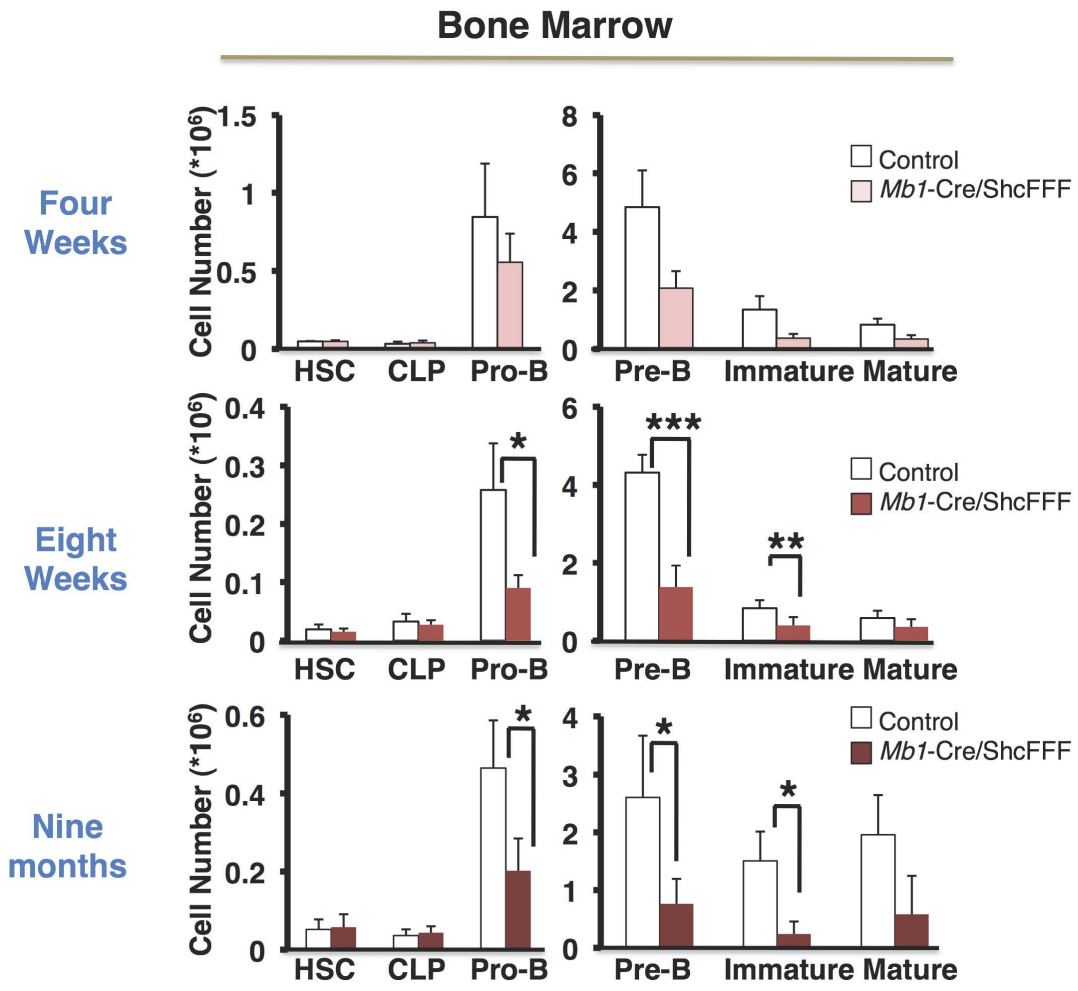
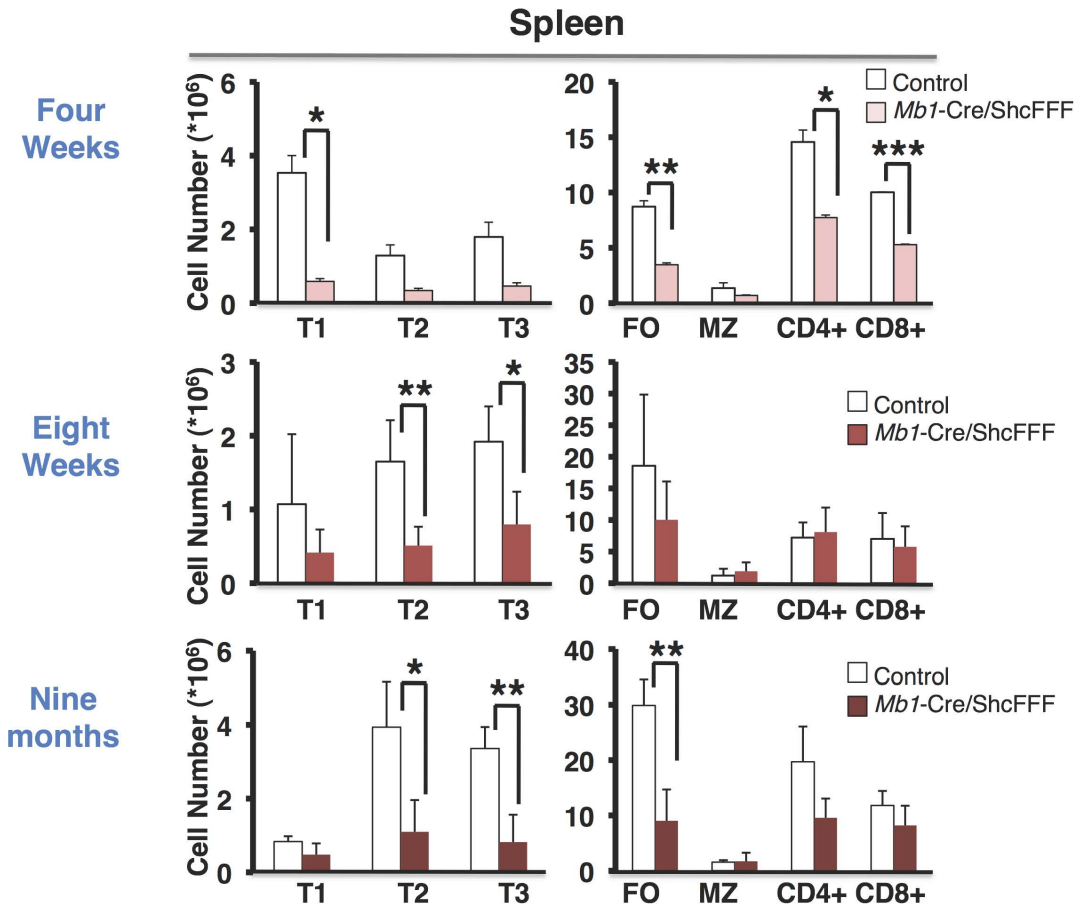

Bone Marrow
Four Weeks
Eight Weeks
Nine months
Cell Number (*10⁶)
HSC
CLP
Pro-B
Pre-B
Immature
Mature
Control
Mb1-Cre/ShcFFF
*
**
***

Figure A2-6. **Effects of aging in *Mb1*-Cre/ShcFFF spleen**. Absolute cell numbers were determined from FACS analysis to identify the indicated lymphocyte subset and total splenocyte counts of *Mb1*-Cre/ShcFFF mice versus littermate controls in (top panel) 4-week-old mice, (middle panel) 8-12-week-old mice, and (lower panel) 9-month-old mice. *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$.

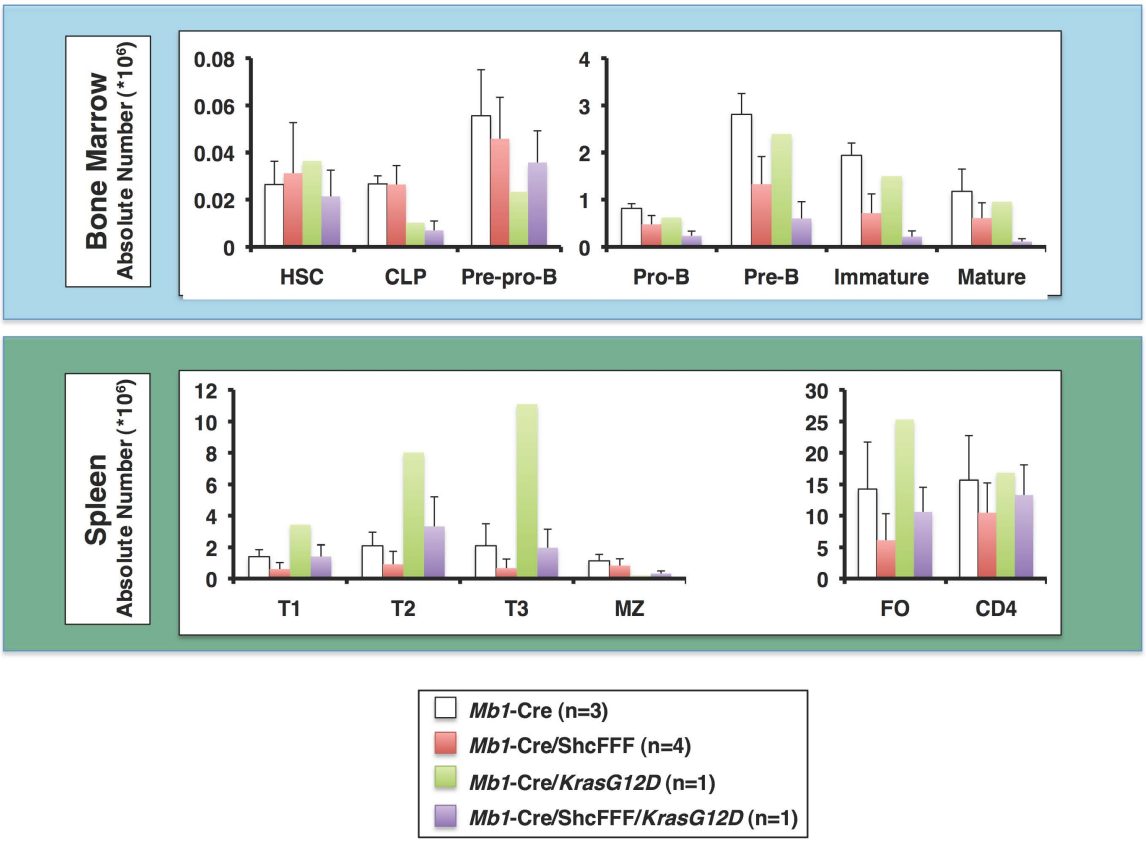

Spleen
Four Weeks
Eight Weeks
Nine months
Cell Number (*10⁶)
T1
T2
T3
FO
MZ
CD4+
CD8+
Control
Mb1-Cre/ShcFFF

Constitutively active Kras does not rescue B development in Mb1-Cre/ShcFFF mice

Since Shc is well established as a signaling intermediate in activation of Ras, I tested the ability of a constitutively active Ras mutant (KrasG12D) to rescue B cell development when conditionally expressed alongside ShcFFF. Somewhat unexpectedly, the presence of KrasG12D appeared to decrease early B cell numbers in the bone marrow (Figure A2-7, top panel). In several B cell subsets, the co-expression of ShcFFF with KrasG12D depressed B cell numbers below that of ShcFFF alone. One possible explanation for this phenotype is that KrasG12D may push cells to differentiate and pass through the developmental stages at a quicker rate than littermate controls. However, previous studies in our lab using the Raf-CAAX transgenic mice have demonstrated that constitutive activation of Raf is unable to rescue the block in T lymphocyte development due to ShcFFF expression. This leaves one to wonder if expression of ShcFFF may affect a signaling pathway that is separate from Ras/Raf activation.

KrasG12D co-expressed with ShcFFF in the spleen is capable of increasing peripheral B cell numbers to control levels (Figure A2-7, lower panel). Although additional mice are needed, it appears that ShcFFF prevents the full expansion of peripheral B cells due to KrasG12D. This could suggest that ShcFFF is disrupting a pathway separate from that activated by KrasG12D.

Figure A2-7. **Conditional expression of RasG12D does not rescue B cell development in *Mb1*-Cre/ShcFFF mice**. Absolute cell numbers were determined from FACS analysis to identify the indicated lymphocyte subset and total bone marrow counts or spleen from the indicated mouse genotype. KrasG12D indicates the presence of a conditionally expressed transgene for constitutively active k-Ras. Total cell numbers for subsets in the bone marrow numbers (top panel) and spleen (bottom panel) are depicted.


Bone Marrow
Absolute Number (*10^6)
0.08
0.06
0.04
0.02
0
HSC
CLP
Pre-pro-B
4
3
2
1
0
Pro-B
Pre-B
Immature
Mature
Spleen
Absolute Number (*10^6)
12
10
8
6
4
2
0
T1
T2
T3
MZ
30
25
20
15
10
5
0
FO
CD4
Mb1-Cre (n=3)
Mb1-Cre/ShcFFF (n=4)
Mb1-Cre/KrasG12D (n=1)
Mb1-Cre/ShcFFF/KrasG12D (n=1)

Mb1-Cre/ShcFFF splenic B cells phosphorylate Erk and flux calcium in response to BCR stimulation.

To directly test Erk activation in ShcFFF-expressing B cells, I stimulated splenocytes from *Mb1*-Cre and *Mb1*-Cre/ShcFFF mice. Since Shc is phosphorylated in response to both BCR and BCR + FcR ligation, I used only anti-IgM $F(ab')_2$ stimulation to stimulate only through the BCR. Rather unexpectedly, I saw a dramatic increase in both the percentage as well as gMFI of phosphorylated Erk in splenic B cells from *Mb1*-Cre/ShcFFF mice compared to littermate controls. A conclusion based on these data is difficult to achieve due to the difference in B cell populations in the spleen of *Mb1*-Cre and *Mb1*-Cre/ShcFFF mice. The increased Erk phosphorylation could be due to a higher percentage of a specific B cell stage in ShcFFF-expressing mice. For example, strong BCR ligation leads to death of immature B cells in the spleen. Perhaps the enhanced Erk activation observed in ShcFFF-expressing mice could be due to an increased presence of immature B cells.

In addition to Erk activation, I tested the ability of splenic B cells from *Mb1*-Cre/ShcFFF mice to release calcium in response to BCR or BCR + FcR ligation when compared to littermate controls. These analyses have the same caveats as listed in the previous figure in that the mixed developmental stages of splenic B cells could have had an effect on the response. $CD19^+$ splenic B cells from *Mb1*-Cre/ShcFFF mice showed diminished calcium flux in response to both BCR as well as BCR + FcR ligation when compared to littermate controls (Figure A2-9, top panels). However, I did not observe a dramatic difference in calcium flux of *Cd19*-Cre/ShcFFF mice during BCR or BCR + FcR stimulation (Figure A2-9, bottom panels). This suggests that the defect in

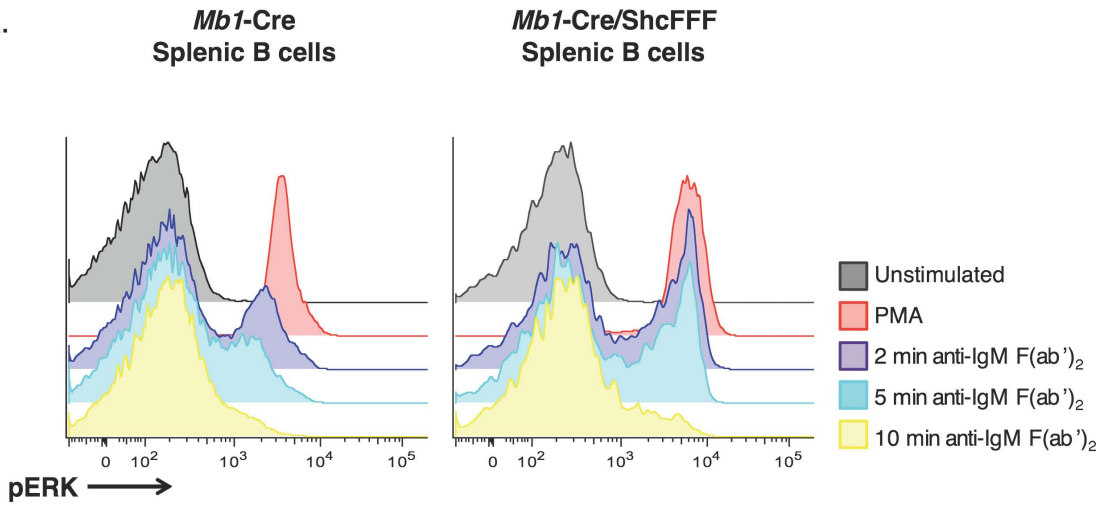
calcium response to stimulation in *Mb1*-Cre/ShcFFF mice may be due to a difference in the available cells during stimulation. Immature B cells show increased calcium flux compared to mature B cells during BCR stimulation [275], and due to their low numbers, they are unlikely to contribute toward the calcium response in *Mb1*-Cre/ShcFFF mice. However, the decreased presence of follicular B cells may contribute toward the decreased calcium response in *Mb1*-Cre/ShcFFF mice.

Peripheral B cells from *Mb1*-Cre/ShcFFF mice display higher levels of CD62L expression than littermate controls yet possess normal levels of CD69 expression (Figure A2-10). This may also be linked to an underrepresented mature B cell population.

Together, these data provide insights into potential mechanisms of Shc signaling during B cell development. B cell development in *Mb1*-Cre/ShcFFF mice is not rescued with age, transgenic expression of Bcl-2, or targeted expression of a constitutively active K-ras protein. Furthermore, Erk activation in *Mb1*-Cre/ShcFFF splenic B cells does not appear to be impaired. Peripheral B cells from *Mb1*-Cre/ShcFFF mice show an impaired ability to flux calcium in response to BCR stimulation. However the ability of *Cd19*-Cre/ShcFFF mice to flux calcium normally suggests that the *Mb1*-Cre/ShcFFF B cell sample may have appeared defective due to reasons other than the ShcFFF protein, such as variations in B cell populations or defects acquired during earlier development. Together, these data provide additional clues to understanding the role of Shc during early B cell development and further support a role for this protein during early B cell development. However, a clear understanding of Shc during early B cell development is lacking, particularly with regards to the mechanism underlying the abnormal cell death in *Mb1*-Cre/ShcFFF early B cells.

Figure A2-8. ***Mb1*-Cre/ShcFFF splenic B cells show increased Erk phosphorylation**. Total spleen from *Mb1*-Cre or *Mb1*-Cre/ShcFFF was collected and used for stimulation. ***A.*** Total splenocytes received PBS (unstimulated), PMA, or BCR stimulation (anti-IgM $F(ab')_2$) for the indicated times during incubation at 37°C (PBS and PMA were incubated 5 minutes at 37°C). After the indicated times, cells were immediately fixed in 2% PFA and analyzed for pERK by FACS. Cells were gated on $B220^+$. ***B.*** The percentage of $pERK^+$ cells under each condition and the geometric MFI (gMFI) of the $pERK^+$ cells from *Mb1*-Cre and *Mb1*-Cre/ShcFFF mice are listed.

A.



B.

	Percent pERK$^+$		gMFI of pERK$^+$	
	Mb1-Cre	**Mb1-Cre/ ShcFFF**	**Mb1-Cre**	**Mb1-Cre/ ShcFFF**
Unstimulated	0.63	0.62	1865	1581
PMA	96.4	95.9	3721	5937
2 min BCR	24.3	40	2350	4253
5 min BCR	17.8	36.7	1980	3816
10 min BCR	5.93	11.9	1680	2460

Figure A2-9. **Splenic B cells from *Mb1*-Cre/ShcFFF but not *Cd19*-Cre/ShcFFF mice show decreased calcium mobilization**. $CD19^+$ splenic B cells were incubated with Indo-1, and their ability to flux calcium in response to BCR (anti-IgM $F(ab')_2$; left panel) or BCR + FcR (intact anti-IgM; right panel) was quantified. Mice represented are *Mb1*-Cre/ShcFFF (top panels) and *Cd19*-Cre/ShcFFF mice (bottom panels). The ratio of calcium-bound Indo-1 to unbound Indo-1 is depicted both before and after addition of the stimulus, indicating the presence of free calcium.

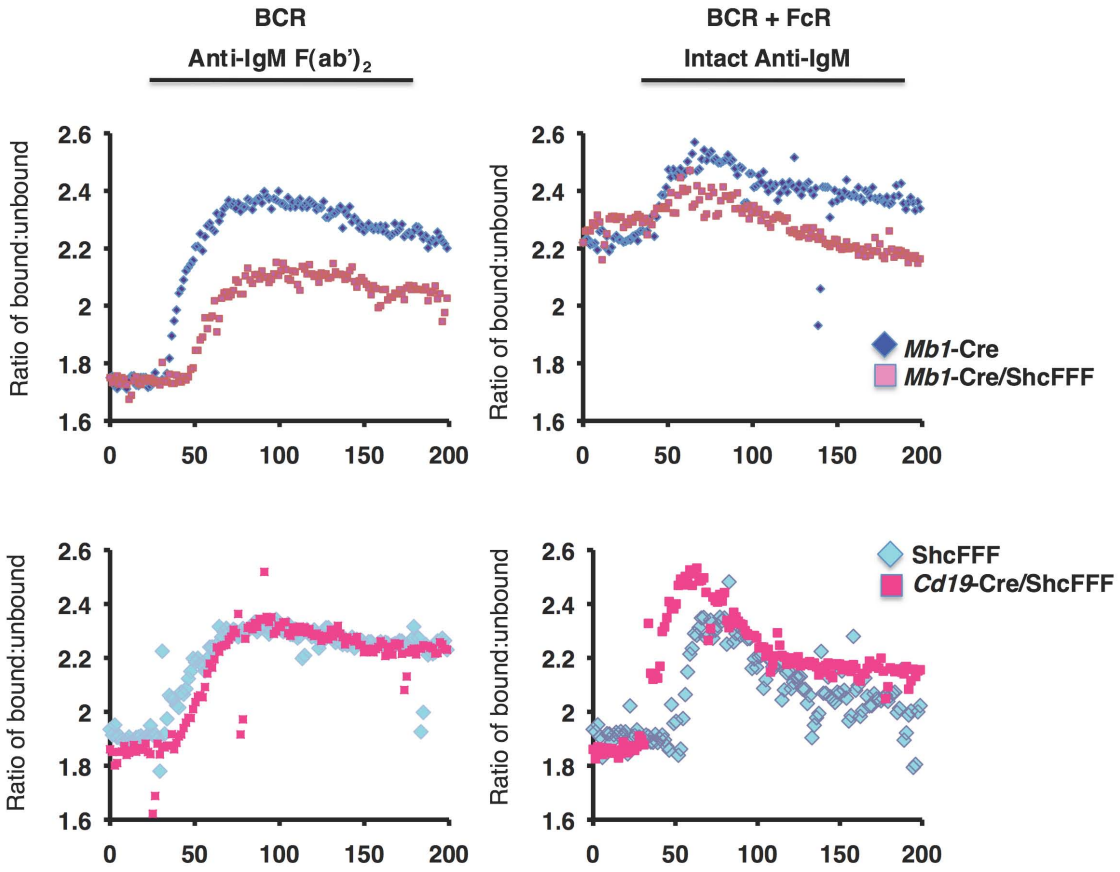
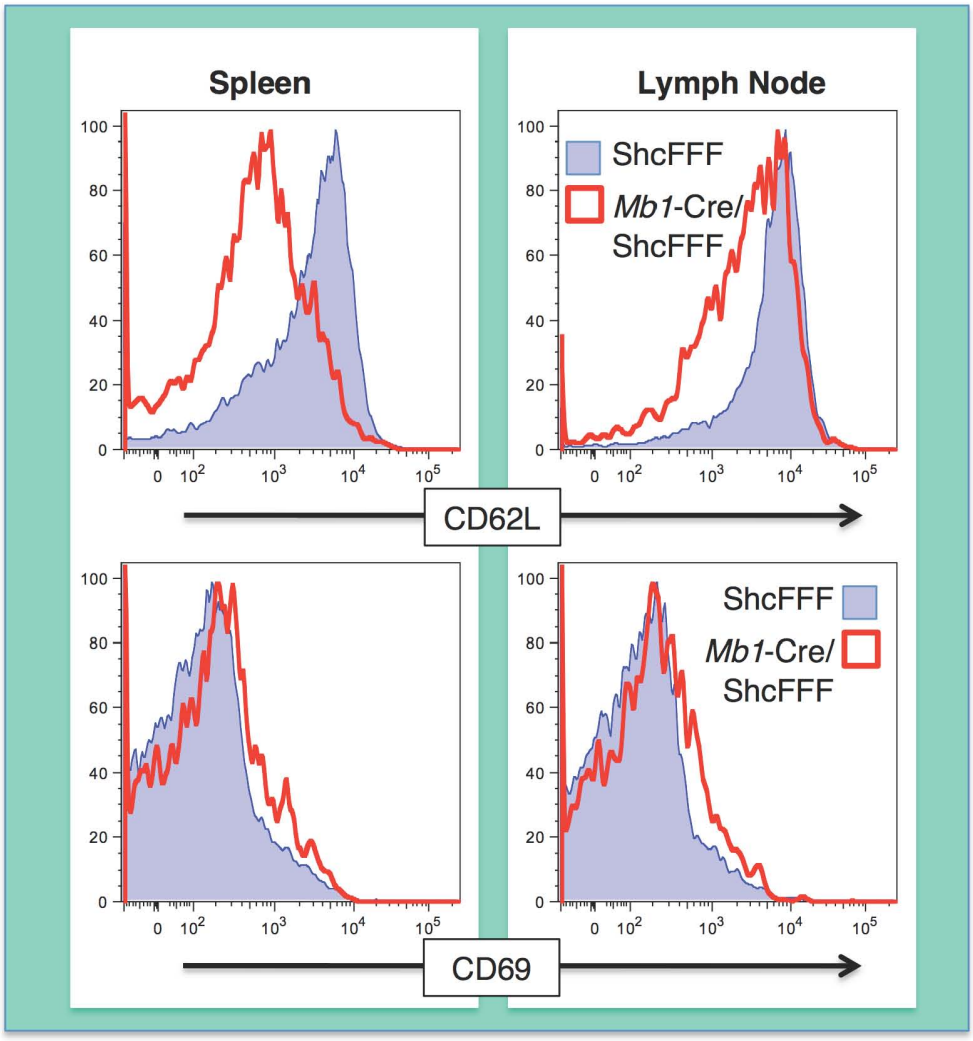

BCR
Anti-IgM F(ab')2
BCR + FcR
Intact Anti-IgM
Ratio of bound:unbound
2.6
2.4
2.2
2
1.8
1.6
0
50
100
150
200
Mb1-Cre
Mb1-Cre/ShcFFF
ShcFFF
Cd19-Cre/ShcFFF

Figure A2-10. ***Mb1*-Cre/ShcFFF peripheral B cells have decreased CD62L expression but normal CD69 expression**. FACS analysis of CD62L expression (top panels) or CD69 expression (lower panels) in the spleen or lymph node of *Mb1*-Cre/ShcFFF and ShcFFF littermate control. FACS analyses were performed on $B220^{+}$ cells from spleen (left panels) and lymph node (right panels).


Spleen
Lymph Node
ShcFFF
Mb1-Cre/
ShcFFF
CD62L
ShcFFF
Mb1-Cre/
ShcFFF
CD69
100
80
60
40
20
0
0
10^2
10^3
10^4
10^5

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