Elucidating Marginal Zone B cell Fate Decisions using Genome-wide Chromatin Accessibility Profiling

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ABSTRACT

The Notch receptor is well described as providing signals that determine cell fate decisions. Of particular interest, is the invaluable signals Notch2 provides for the development of marginal zone B cells, which, upon a defect in Notch2-sginaling, are unable to develop. However, whether Notch2 is involved in providing signals for the subsequent maintenance and survival of marginal zone B cells is relatively undescribed. Here, we demonstrate that indeed Notch-signaling is significant for the survival of not only marginal zone B cells, but their precursors as well. By using a novel approach for Assaying for Transposase-accessible Chromatin (ATAC-seq) we were additionally able to demonstrate that Notch-signaling is involved in shaping the chromatin landscape of not only marginal zone and precursor B cells, but follicular B cells as well, a cell type in which Notch-signaling is not essential for development. In marginal zone and marginal zone precursor cells, we show that Notch-signaling is potentially involved in maintaining large regions of enhancer clusters, known as stretch enhancers, highly regulated regions of the genome important in directing cell-specific functions and identity. Additionally, through motif enrichment analysis, we uncover a possible role for the transcription factor CTCF in promoting the survival of a subset of marginal zone B cells in an environment depleted of Notch-signaling. The work presented here invites further research into the mechanism through which the Notch receptor is both providing survival signals for marginal zone and marginal zone precursor B cells and shaping the chromatin landscape of these cells to maintain their function.

INTRODUCTION

The field of immunology has long been divided into two distinct but overlapping realms: innate immunity and adaptive immunity. Innate, or nonspecific, immunity, is often characterized as the body's first line of defenders against possible invaders. Responding within the first few hours of infection, this line of defenders includes the skin and the epithelial and mucosal linings of the respiratory and gastrointestinal tracts which provide a physical and chemical barrier against pathogen entry, phagocytic cells such as neutrophils and macrophages, dendritic cells (DCs), mast cells, natural killer cells (NK), all which are crucial in fighting infectious pathogens, and blood proteins which mediate inflammation[1]. A key component of innate immunity is that this first line of defense exists even before the onset of infection, and thus can rapidly respond upon stimulation by infectious agents. The mechanism with which the innate immune system responds to invading microbes is fairly consistent regardless of the pathogen, as its receptors, namely Toll-like receptors (TLRs) expressed mainly on dendritic cells and macrophages, respond to common conserved structural elements across various microbes. Therefore, even repeated exposure to the same microbe results in the same mechanism, length, and intensity of innate immune response[1].

On the other side of the immunity spectrum is adaptive, or acquired, immune system, aptly termed because its ability to adapt a specific, specialized response by recognizing a wide variety of both microbial and nonmicrobial substances known as antigens. Responding slowly within days of infection and secondarily to innate immunity, the master regulators of the adaptive immune system are white blood cells known as lymphocytes, which are composed of two main populations: T lymphocytes (T-cells) and B lymphocytes (B-cells). By secreting molecules known as antibodies, B-cells are the mediators of humoral immunity, primarily

targeting toxins on the external surface of cells. Antibodies secreted by B-cells can identify foreign antigens, neutralize their infectious properties, and target them for elimination by phagocytes or factors in the complement system. On the other hand, T-cells are regulators of cell-mediated immunity, primarily targeting microbes internally residing in cells and promoting the destruction of infected cells to prevent the spread of infection[1]. Whereas the innate immune system mounts consistent responses to various microbes, the adaptive immune system can mount a wide diversity of specific responses, as lymphocyte receptors are capable of distinguishing among epitopes, or parts of antigens, from a grand selection of distinct antigens. In fact, the estimated number of epitopes an individuals lymphocytes can recognize is between 10^7 and 10^9, representing the vast and diverse capabilities of the adaptive immune system. In contrast to the innate immune system, upon repeated exposure, the adaptive immune system will mount a response with increased intensity and defensive capability, a phenomenon regarded as memory[1].

Though often recognized as distinct dimensions of immunity, the innate and adaptive immune responses are widely interconnected. While the innate immune system provides the initial defense against invaders, it also provides danger signals to the adaptive immune system in order for it to mount a specialized secondary response. For example, DCs and macrophages from the division of innate immunity sense pathogen-associated molecular patterns (PAMPs), conserved epitopes on microbes, respond via pattern recognition receptors (PRRs), and instruct B and T-cells of the adaptive immune system to mount a temporally delayed attack via somatically recombined T-cell receptors (TCRs) and B-cell receptors (BCRs)[2]. As a result, the adaptive immune system often enhances the initial immune protection provide by the innate immune system in a further specialized manner[1].

Immunity is undoubtedly characterized by a vast repertoire of agents that mediate sequential and coordinated action against infection. In contrast to the immensity of the immune system, the work presented here focuses on a small, but significantly important subset of humoral adaptive immunity known as marginal zone (MZ) B-cells, which over the years have revealed to be a particularly interesting population of cells that blur the conventional boundaries between innate and adaptive immunity.

Follicular vs. Marginal Zone B cells

The humoral immune response mounted by B cells upon activation generally involves a variety of effector functions, including antigen presentation, differentiation into plasma cells that secrete the clonally selected antibody to fight the invader, and generation of memory B-cells which remember the infectious agent and provide a reservoir for rapid antibody generation upon future infection by that very agent.

Most mature B cells that mediate this typical adaptive immune response are known as recirculating Follicular (FO) B cells. These cells primarily express monoreactive BCRs encoded by extremely diversified VDJ genes. These distinct receptors allow FO B cells to engage in highly specified interactions with pathogenic invaders. FO B cells mainly reside in B cell follicles of secondary lymphoid organs, such as the lymph nodes and spleen. As these follicles are adjacent to T cell zones, this residence allows activated FO B cells to be astutely situated to migrate towards and interact with activated T cells, thereby initiating the germinal center (GC) reaction and mounting T cell dependent B cell proliferation, affinity maturation, class-switching, and differentiation into either memory B cells or high affinity antibody generating plasma cells characteristic of the adaptive immune response. However, FO B cells can also recirculate

through the bone marrow where, due to their organization around sinusoids, they are regularly exposed to blood-borne pathogens, to which they can respond in a T cell independent manner. Though these cells are recirculating, they have a defined lifespan that lasts only up to several weeks[2,4].

Though most B lymphocytes, as master regulators of the humoral adaptive immune response, are characterized by their highly specific antigen receptors, there are select subsets of B cell populations whose BCRs are encoded by semi invariant or inadequately diversified VDJ genes. As a result, veering from their representative adaptive immune function, these B cells have rather "innate-like" phenotypes stemming from their polyreactive BCRs that can recognize multiple microbial antigens, similar to the activity of TLRs[2,3]. An increasingly characterized and fascinating population of these B cells includes Marginal Zone (MZ) B cells, self-renewing cells that's lifespan can last as long as that of the host itself. Characterized mainly in rodents, MZ B cells are immobile and reside in the spleen, mainly in the outer white pulp, separated from the red pulp with a loose-endothelial region known as the marginal sinus, a location constantly exposed to microbial antigens in slow moving blood received by the spleen. In humans, MZ B cells are also found in areas with a cellular composition similar to that of the spleen, such as the subcapsular sinus of lymph nodes and subepithelial regions of mucosa-related lymphoid tissues, and in the peripheral blood, suggesting they might be recirculating cells as well[4].

MZ B cells are particularly interesting, because they respond much more vigorously to blood-borne pathogens than FO B cells[3,5]. With high levels of CD21 expression, a receptor involved in the complement system, MZ B cells further the transport of immune complexes to the splenic follicles, facilitating follicular dendritic cell (fDC) capture and therein presentation to FO B cells. Additionally, MZ B cells also express high levels of CD1d, allowing the presentation

of lipid antigens to invariant natural killer T cells (iNKT). Furthermore, MZ B cells intrinsically appear to be in a pre-activated, "memory-like" state, differentiating rapidly (in a matter of hours) into plasma cells upon in-vitro activation by LPS, anti-CD40, or IL-4[6]. Thus, during the temporal gap between the onset of infection and conventional FO B cell high-affinity antibody response characteristic of the adaptive immune system, these MZ B cell generated plasma cells can provide a primary wave of humoral immunity by producing comparatively short-lived, low-affinity antibodies. Because of their uncharacteristic phenotype, MZ B cells present an intriguing potential target for immune regulation and therapeutics.

Marginal Zone B-cell Development

As FO and MZ B cells have widely different phenotypes, residence, and associated functions, much research has been conducted in mice to investigate both the sequence of fate decisions naive B cells make to eventually differentiate into either mature recirculating FO B cells or non-recirculating MZ B cells, and then the signals required for each cell population's maintenance.

Both FO and MZ B cells are members of the B-2 B cells lineage and develop from hematopoietic stem cells (HSCs) in the bone marrow. In the B cell lineage pathway, HSCs differentiate into lymphoid progenitors which give rise to B lymphocyte progenitors that can then differentiate into immature Pre-Pro-B cells, Pro-B cells, and then Pre-B Cells in an antigen-independent manner. During this stage of development, positive selection for ligand binding occurs as Pro-B cells first rearrange genes at their immunoglobin heavy chain (Ig H) locus. Once Pro-B cells have successfully and productively rearranged their heavy chain and express a properly assembled pre-BCR on the cell surface, they proliferate into Pre-B cells which then

undergo immunoglobin light chain (Ig L) gene rearrangement at the Igκ locus. With successful Igκ rearrangement, Pre-B cells finally develop into immature B cells with a fully assembled IgM molecule on the cell surface[7]. However, many of these immature B cells are strongly self-reactive, and thus undergo negative selection, clonal deletion, or further BCR editing with new Igκ or Igλ gene rearrangements to produce immature B cells that are weakly or not self-reactive. Naïve B cells then mature further through transitional (T1 and T2) B cell stages before becoming mature FO or MZ B cells. T1 B cells are found in the bone marrow and spleen and are characterized by the surface markers $IgM^{hi}IgD^{lo}CD21^{lo}CD23^{lo}$ and the inability to recirculate[8]. Once T1 B cells gain surface IgD and CD23 and the ability to recirculate, they are known as T2 B cells. T2 B cells can then reside in the bone marrow and mature into FO B cells, but most of these naïve B cells respond to signals taking them to the spleen, where they can then mature into either FO or MZ B cells[9].

T2 B cell maturation in the spleen is of particular interest, because it is in the spleen that naïve B cells receive signals to commit to either FO or MZ B cell fate, resulting in cells with starkly different phenotypes. For instance, on the most polarizing level, T2 B cells can make the decision to become FO B cells, which allows them the ability to recirculate and migrate among secondary lymphoid organs, but limits their lifespan to only a few short weeks. On the other hand, T2 B cells can actively decide to differentiate into MZ B cells, physically limiting their residence to the spleen, but fairly elongating their lifespan to that of the host itself. Much research that has elucidated these cell fate decisions has been done by creating transgenic mice and observing the resulting mature B cell phenotypes. These studies have revealed classes of mutations that result in a relative loss of MZ B cells, designating signals required for MZ B cell generation and maintenance. The classes of mutations more relevant to a deficit in MZ B cell

development were discovered to be: (1) ones that result in enhanced BCR signaling, (2) ones in the B cell activating factor receptor (BAFFR) and the classical NF-κB pathway, (3) ones in the Notch2 signaling pathway, and (4) ones linked to integrin or chemokine activation that may contribute to MZ B cell retention in the marginal zone[5]. Of the mutations discussed, the ones most relevant to this study are those that result in a deficit in Notch2-signaling, and thus will be described in further detail.

Notch2 Signaling

Of the regulators involved in MZ B cell differentiation, of particular interest is the pivotal role of Notch2 signaling in MZ B cell commitment and FO B cell suppression. Notch encoded receptors are single-pass transmembrane proteins. This type-1 transmembrane protein is characterized by a functional extracellular ligand-binding domain (NECD) non-covalently attached to a transmembrane (TM), and intracellular (NICD) domain. Upon receiving signals from cells via members of the Delta-like (DL1-4) and the Jagged (JAG1 and JAG2) ligands, NECD is cleaved from TM-NICD by metalloproteninase and disnitgerin-10 (ADAM10), bound to the ligand, endocytosed and recycled in signal-sending cells. Signal transduction occurs when γ-secretase cleaves NICD and releases it from TM[10]. In the signaling pathway vital for MZ B cell commitment, NICD then translocates to the nucleus and forms a complex with recombinant binding protein suppressor of hairless (RBP-Jκ) and Mastermind-like 1 (MAML1). This complex then allows for the transcription of Notch target genes, which together with classical NF-κB regulated by B cell activating factor (BAFF) and its receptor (BAFFR) interactions, seemingly instructs MZ B cell development and commitment to MZ B cell Fate[11,12,13] (Figure 1).

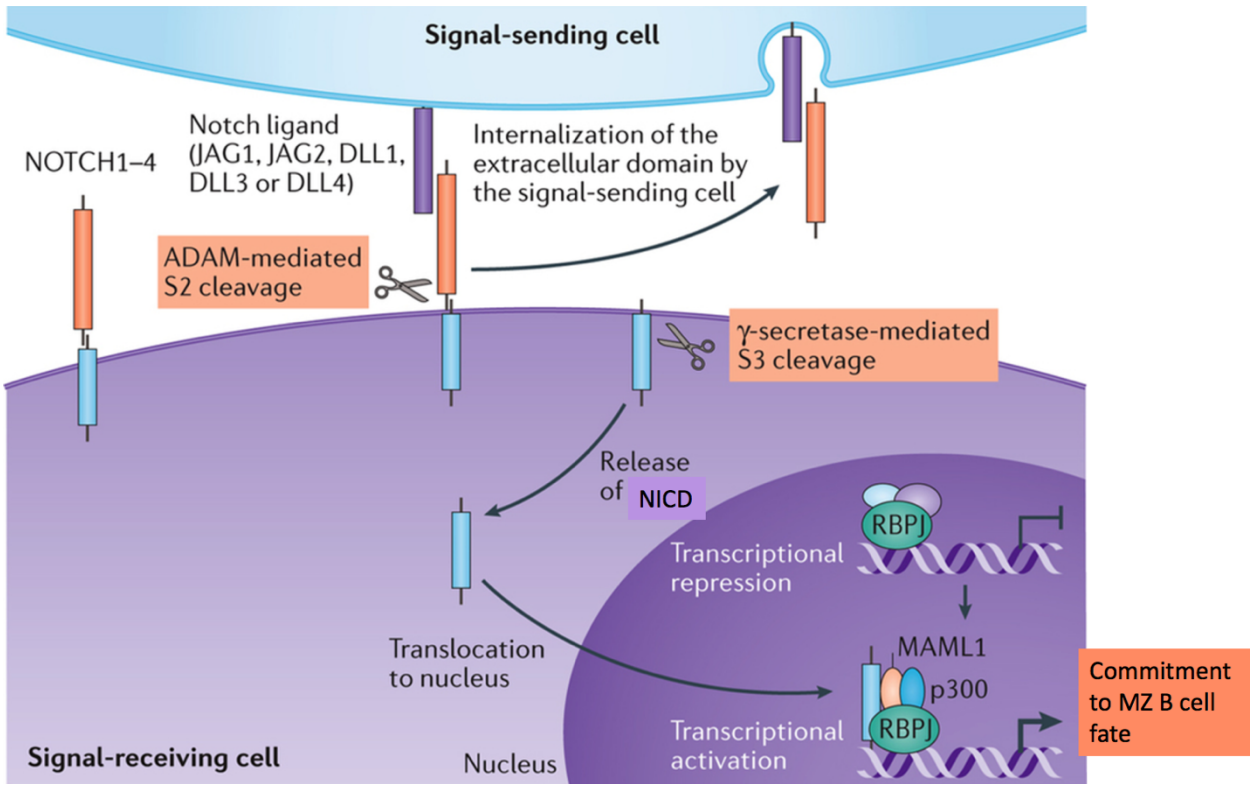

Figure 1. The role of Notch2-signaling in marginal zone B cell fate commitment. Upon cleavage by γ-secretase, NICD translocates to the nucleus, forms a transcriptional complex with RBP-Jκ and MAML1, and in conjunction with other signals, instructs MZ B cell fate commitment (Adapted from Nowell & Radtke, 2017[14]).

The involvement of Notch2 in MZ B cell fate commitment was discovered in Cre-loxP-mediated site-specific recombination Notch2 conditional knockout mice[14]. In the conditional knockouts, while FO B cell populations appeared normal, MZ B cells and CD1d^{hi} MZP cells were absent. In addition, in mice generated with a selective deficit in RBP-Jκ, researchers observed a loss of MZ B cells while likewise observing an increase in FO B cell populations[15]. Because the deficit in RBP-Jκ did not appear to have significant effects on B cell maintenance, survival, plasma cell generation, or activation, these results further supported the hypothesis RBP-Jκ interaction with Notch2 serves a lineage commitment function to the MZ B cell phenotype. Similar phenotypic results were observed in mice with a mastermind-1 deficiency,

which diminished the development of MZ B cells[16]. Furthermore, researchers observed that when Msx2-ineracting nuclear target protein (MINT), a suppressor of Notch signaling that competes with NICD for binding to RBP-Jκ, was knocked out in embryos their fetal liver cells were transplanted into sublethally irradiated $RAG^{-/-}$ mice, splenic MZ B cells increased threefold[15]. Finally, Delta-like 1 (DL1) proved an indispensable ligand for Notch2-signaling mediated MZ B cell development, as DL1-null mice failed to generate a significant population of $IgM^{hi}IgD^{lo}$ MZ B cells.

Evidence demonstrating that DL1 is an essential ligand for Notch2 signaling in MZ B cells is further supported by research demonstrating that DL1 is expressed on both the luminal side of the red pulp in the spleen and the marginal zone[16]. Though this DL1-Notch2 interaction is inherently weak, researchers demonstrated that the DL1-induced Notch2 activation in MZPs is enhanced by lunatic and manic glycosyltransferases by adding M-acetyl glucosamine groups to O-linked fucose residues on Notch2. This modification and enhanced interaction allows for developing MZP B cells to compete for limited DL1 expressed on fibroblastic reticular stromal cells in the spleen[17] and fully differentiate into MZ B cells.

Though it was previously proposed weak BCR signals may allow T2 B cells to be permissive Notch2 instruction towards MZ B cell fate, while strong BCR signals render them resistant to DL1-mediated Notch2 activation[5], whether BCR signaling controls Notch2 receptiveness in these cells is relatively unknown. In addition, questions remain regarding which transitional B cells respond to Notch2 signaling and in what manner. For instance, does Notch2 indeed provide differentiation signals for transitional cells to become MZ B cells, or does it provide survival signals for MZ B cell populations, which, when lost, results in a decrease in cell population numbers?

Therefore, though Notch2 signaling is regarded as an invaluable activator of MZ B cell development an interesting question that remains is about its potential role in the survival and maintenance of the fascinating B cell population. A study conducted by Moriyama, et al. (2008) found that upon treating adult mice with an antibody blocking DL-1, pre-established MZ B cell populations became depleted in the spleen[18]. Because DL-1 is a known ligand for Notch-signaling, this study implicated that there may be a role of Notch2-signaling in regulating the maintenance and survival of MZ B cells in addition to activating a cell fate decision.

We seek to further address this relatively unknown role of Notch-signaling by inhibiting signal transduction pathway in mice and observing the effects on MZ, MZP, and FO B cell populations. Because the molecule responsible for the signal transduction of Notch-signaling is NICD, it would be interesting to generate antibodies against NICD in order to assess the affects of Notch-signaling inhibition on MZ B cell populations. However, NICD has two cellular conformations, one in which it remains bound to TM and NECD prior to activation, and another in which it is free to translocate to the nucleus and regulate transcription, thus, it is difficult to generate antibodies specifically targeting free NICD to block signal transduction. Thus, here we propose using a γ-secretase inhibitor to prevent NICD cleavage and resulting nuclear translocation in order to observe the effects a lack of Notch-signaling on existing B cell populations.

Genome-wide Chromatin Accessibility Profiling

Though most studies investing the drivers of cell fate decision and survival have been investigated through transgenic mouse studies, these studies have been widely based on observing phenotypes of knockout mice and making predictions about sequence of signaling

events that lead to such phenotype by concentrating on specific genes. Here, we propose different method for investigating drivers of cell fate and survival, specifically Notch2-signaling and MZ B cell survival, relying on over 30 years of advances in biotechnology that have allowed researchers to observe patterns in genome-wide chromatin accessibility. These advances, as I will describe below, will allow us to better make unbiased characterizations about noncoding functional elements in genomes involved in highly cell-selective regulation.

DNA in eukaryotic genomes is tightly packaged into chromosomes, and the hierarchical structuring of this packaging is centrally involved in gene regulation[19]. For instance, regions of tightly would DNA around histone proteins into nucleosomes, known as heterochromatin, is associated with transcriptionally inactive regions. This highly ordered structuring is characteristic of repeating units of eight histone proteins and approximately 200 nucleotide base pairs of wound DNA. Regions of transcriptionally active DNA, on the other hand are characteristic of decondensation of heterochromatin and nucleosome structure in a series of events that eventually leads to euchromatin, lightly wound DNA, freeing transcriptional regulatory elements and affording gene expression.

A DNA regulatory element revealed in regions of accessible chromatin of particular interest are regions known as “superenhancers” or “stretch enhancers”, regions of DNA characterized by two or more enhancer sites approximately 10kb apart located around TSS elements[20]. Algorithmically clustered, these concentrated enhancer regions are highly enriched for factors associated with enhancer gene expression regulatory activity, including Pol II, chromatin factors such as cohesin, and histone modifications H3K27ac and H3K4me2 associated with accessible chromatin[21]. Because they involve regions of the most densely regulated

chromatin, stretch enhancers provide insight into the signals of utmost importance for cell-specific function, generation, and maintenance.

Because these open, or accessible, regions of DNA clearly contain information about cell-specific gene expression, years of research has focused on assaying these regions and chromatin structure to characterize cell identity. Major insights about the information encoded in these open regions of DNA have resulted from high-throughput, genome-wide methods that have allowed researchers to access these open regions[22], further understand nucleosome positioning[23], and reveal transcription factor occupancy,[24] elucidating effects of chromatin remodeling.

DNase I Hypersensitivity, Nucleosome Positioning, and Transcription Factor Occupancy

One of the premier methods established to assess active regulatory elements of DNA, including promoters, enhancers, silencers, and insulators, relied on the identification of regions hypersensitive to DNase I cleavage[25]. DNase I is an endonuclease that can nonspecifically bind to single and double stranded DNA, releasing 5'-phosphorylated di-, tri-, and oligonucleotide products[26]. Because regions of accessible DNA are relatively void of hierarchical nucleosome structures, these regions are available for easy digestion by DNase I and are known as DNase I hypersensitive (HS) sites and allow researchers to make predictions about the relationship between available sites and cell identity control based on information inferred about chromatin-binding proteins that lie between HS sites. Most recently, the Encyclopedia of DNA Elements (ENCODE) Project used high-throughput methods to categorize functional regulatory elements encoded in the human genome[27]. The project revealed that these DNase I HS sites identified in 1% of the human genome were markers for non-protein-coding transcripts, transcription start

sites (TSS), regulatory sequences symmetrically distributed around transcriptionally active regions, and transcription binding sites.

Traditionally, DNase I HS sites were individually identified using Southern Blotting detection[28], but the technique is fairly experimentally demanding and low-throughput, limiting detection to only small regions of accessible chromatin. Thus recently, researchers developed a high-throughput sequencing method for identifying DNase I HS sites across the entire genome by taking approximately 50 million cells, digesting them with DNase I, and sequencing the resulting fragments with next-generation sequencing, providing high-quality resolution of accessible human chromatin[29,30].

Veering slightly from DNase I hypersensitivity and characterizing solely highly accessible regions of chromatin, the era of uncovering epigenetic information from the hierarchical nature of chromatin has also focused on the positioning of nucleosomes in DNA and its relationship with gene expression. Because covalent histone modifications such as phosphorylation, ubiquitination, methylation, and acetylation have been demonstrated to play significant roles in gene expression by affecting nucleosome stability and ability to form hierarchical structures, therefore controlling accessible regions of chromatin available for transcription[31], the relative ordering of nucleosomes on chromatin is of particular interest to researchers. For instance, after assessing genome-wide patterns of histone methylation in human $CD4^+$ T cells and observing subpeaks of H3K4me3 (signifying three methyl groups added to K4 on H3 histone proteins) about 150bp apart[32], Schones, et al. (2008), sequenced ends of the observed mononucleosome-sized DNA fragments isolated from MNase-, an endonuclease that digests linker DNA between nucleosomes, digested chromatin. Using this method, they were able to effectively map genome-wide nucleosome positions in the human genome and make

observations such as that nucleosomes tended to be positioned in a highly ordered fashion around transcriptional start sites (TSS) in regions of expressed genes, with the RNA Polymerase II (Pol II) promoter region exposed in a nucleosome-free region of about 200 bp. Thus, the researchers were able to make inferences such as that the binding of Pol II may prevent nucleosomes from accessing the promoter region of expressed genes. Therefore, they were able to reveal a highly intimate relationship between the nucleosome structure and the regions of the DNA it regulates.

In addition to identifying genome-wide DNase I HS sites and nucleosome positioning, it has been of particular interest to researchers the binding patterns of transcription factors to accessible regions of DNA and how this information distinguishes gene expression in cell populations. Often used in conjunction with DNase and MNase-seq, a method known as chromatin immunoprecipitation with sequencing (Chip-seq) assays for DNA protein binding *in vivo* by crosslinking DNA-binding proteins to DNA with formaldehyde treatment. After sonicating the cross-linked DNA into small fragments, researchers are then able to enrich for fragments bound to proteins or nucleosomes immunoprecipitated by specific antibodies and sequence them[33]. Chip-seq allows researchers to precisely map protein-binding sites on DNA, providing invaluable information about targets for transcription factors and enhancers genome-wide.

ATAC-seq

Finally, the method for understanding chromatin accessibility and associated functional elements most recently entering the field is known an Assay for Transposase Accessible Chromatin using high-throughput sequencing (ATAC-seq)[34]. This method was developed by Buenrostro, et al. (2013) as a response to the time-consuming and requirement for millions of cells as starting material traits of the aforementioned methods. Pivotal in their argument for a

novel method was the idea that with such high cell numbers, heterogeneity in cell populations may be over averaged. Additionally, a requirement for such significant starting material limited the methods to *ex vivo* cell culture applications, disallowing an understanding of the chromatin landscape in from *in vivo* contexts. Thus, Buenrostro, et al. (2013) developed ATAC-seq, which allowed them to assay regions of accessible chromatin in hours with as little as 50,000 cells.

Using hyperactive Tn5 transposase, an enzyme used to catalyze the *in vitro* integration of synthetic oligonucleotides with known sequences into target DNA, ATAC-seq allows researchers to simultaneously fragment and tag accessible regions of chromatin with sequencing adapters, a process known as tagmentation[35]. The wild type Tn5 transposition occurs in a "cut and paste" manner, in which the transposon is cut from the donor DNA and pasted into target DNA[36] (Figure 2a). While WT transposon DNA is flanked by two intervened insertion sequence elements (IS50), each containing two 19bp end sequences required for function, these end sequences have relatively low activity, and were replaced by hyperactive mosaic end sequences (ME) and. Because the transposition is also relatively infrequent *in vivo,* the Tn5 protein also acquired several hyperactive mutations. Thus, reliant on the fact that the intervening donor DNA is long enough to bring the ME together, forming an active Tn5 transposase homodimer, hyperactive Tn5 transposase can nonspecifically bind regions open chromatin, as steric hindrance prevents binding from tightly wound chromatin, and catalyze the insertion of free 19bp ME to the 5' ends of target DNA[37]. Nextera-sequencing primers with a sample-specific barcode can then be used to PCR amplify the resulting tagmented DNA and create sequencing libraries to map tagmented DNA to the genome (Figure 2b).

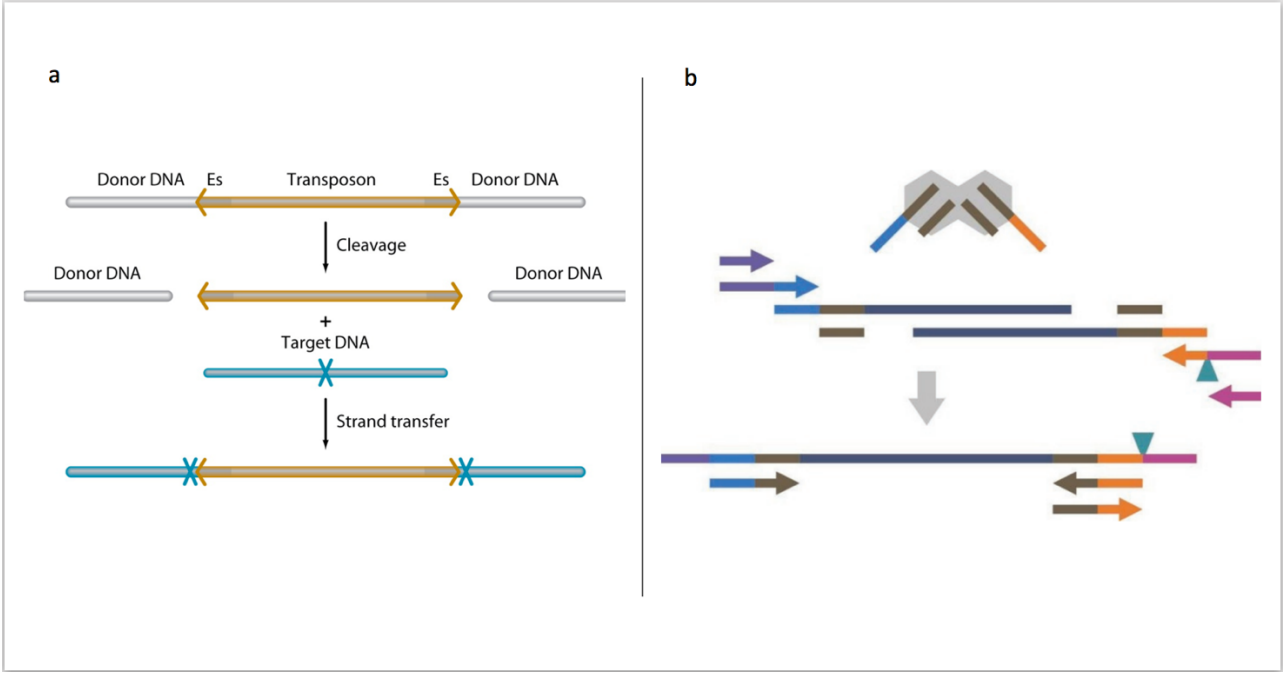

Figure 2. Model of Tn5 transposition. a) Cut and paste mechanism of transposition. Transposon DNA is cleaved from Donor DNA and inserted into target DNA (Adapted from Reznikoff, W.S. 2008). b) Hyperactive Tn5 transposase-mediated ME insertion into target DNA. Navy represents the genomic DNA tagmented by Tn5 transposase represented by hexagon. The inserted ME adaptors are shown in blue and orange and the PCR-incorporated Nextera sequencing primers are shown in magenta and purple. The sample-specific barcode is represented by the teal triangle (Adapted from Adey, et al. 2010).

With their robust method for assaying regions of open chromatin, Buenrostro, et al. (2013) demonstrated the ability to simultaneously integrate previous methods and observe transcription factor occupancy, nucleosome positioning, and overall accessibility of chromatin genome-wide, a vital tool in the growing field of epigenomic research.

OBJECTIVES

Our lab has previously observed global changes in regions of accessible chromatin in FO and MZ B cell populations using ATAC-seq (unpublished data). Because Notch2 is a known regulator of MZ B cell commitment, we wanted to assess how inhibiting Notch signaling with a γ-secretase inhibitor would (1) affect MZ and MZP B cell maintenance and (2) affect chromatin accessibility in these cells, a direction of research previously undefined.

We hypothesized that Notch2-signaling was important in regulating both MZ and MZP B cell maintenance, and thus by treating mice with a γ-secretase inhibitor, we would see a reduction in both cell populations. Additionally, we expected that a γ-secretase inhibitor would cause changes in the regulatory landscape and accessible chromatin of surviving MZ and MZ B cells. We expected that because the intracellular domain of the Notch receptor is typically associated with transcriptional activation, in the absence of Notch-signaling, regions of chromatin in MZ and MZP B cells would most likely become decreasingly accessible. By further analyzing these expected differences in chromatin accessibility, particularly in previously described enhancer regions, we hoped to expand the investigation into the role of Notch2-signaling in MZ B cell maintenance in a novel manner.

METHODS AND MATERIALS

Notch-Signaling Inhibitor Treatment

8 C57BL/B6 mice were selected for the experiment. All mice were subsequently weighed and placed in either the control group or Notch-inhibitor treated groups. 4 mice orally received 100μL/kg of phosphate buffered saline (PBS) for 5 days (control), while 4 mice received γ-secretase inhibitor LY411575 (LY-GSI, 10 mg/kg, Selleckchem) for 5 days. On day 5, the spleens of the mice were collected for FACS analysis.

Flow Cytometry

Mouse splenocytes were hemolyzed with ammonium-chloride-potassium (ACK) buffer and stained with fluorescently conjugated antibodies and analyzed on a BD LSR II flow cytometer. The following antibody clones were purchased from Biolegend for this study: CD19 (1D3/CD19), CD1d (IB1), CD21 (7E9), IgM (AF6-78), and IgD (11-26.c.2a). Dead cells were excluded by 7AAD staining. All cells were gated as Live and $CD19^{+}$. MZ B cells were gated as $IgM^{hi}IgD^{lo}Cd21^{+}CD1d^{+}$, MZP B cells were gated as $IgM^{hi}IgD^{hi}Cd21^{+}CD1d^{+}$ and FO cells were gated as $IgM^{lo}IgD^{hi}$.

Fast-ATAC-seq

An optimized version of the Assay for Transposase-accessible Chromatin with high Throughput Sequencing (ATAC-seq) was performed according to Corces, et al. (2016)[38] on the FO, MZ, and MZP cells collected via flow cytometry. 50,000 sorted cells in FACS buffer were

pelleted by centrifugation at 500g RCF for 5 min at 4°C in a precooled fixed-angle centrifuge. All supernatant was removed and the cells were resuspended in 100μL PBS and pelleted again by centrifugation. All supernatant was removed while avoiding disturbing the cell pellet, which was not visible at this time. The cell pellet was disrupted and resuspended in fifty microliters of transposase mixture (25μL of 2X TD buffer (Nextera), 2.5μL of TDE1 (Illumina), 0.5μL of 1% digitonin, and 22μL of nuclease-free water). The suspension was thoroughly mixed by pipetting. The transposition reactions were incubated at 37°C for 30 min in an Eppendorf ThermoMixer with gentle agitation at 300 rpm. The transposed DNA was purified using a QIAGEN MinElute PCR Purification Kit and eluted in 10μL of nuclease-free water. Purified DNA was stored at -20°C before continuing.

Transposed DNA was amplified according to Buenrostro, et al. (2015)[39]. 10μL Transposed DNA, 10μL nuclease-free water, 2.5μL 25μM Custom Nextera PCR Primer 1, 2.5μL 25μM Custom Nextera PCR Primer 2 (A full set of primers used are litsted in supplementary Table 1), and 25μL NEBNext High-Fidelity 2X PCR Master Mix were combined in PCR 8-tube strips. Thermal cycle was as follows:

1 cycle of 72°C for 5 min, 98°C for 30 sec

5 cycles of 98°C for 10 sec, 63°C for 30 sec, and 72°C for 1 min

In order to reduce GC and size bias and allowing us to stop amplification before saturation, the appropriate number of remaining PCR cycles was determined using qPCR. 5μL of the previously amplified DNA, 4.41μL nuclease-free water, 0.25μL 25μM Custom Nextera PCR Primer 1, 0.25μL 25μM Custom Nextera PCR Primer 2, 0.09μL 100X SYBR Green I, and 25μL

NEBNext High-Fidelity 2X PCR Master Mix were combined in a 96-well plate. Thermal cycle was as follows:

1 cycle of 98°C for 30 sec

20 cycles of 98°C for 10 sec, 63°C for 30 sec, and 72°C for 1 min

In order to calculate the additional number of cycles needed, the Rn vs. cycle was plotted and the cycle number that corresponded to ¼ of maximum fluorescent intensity was determined.

The remaining 45μL PCR reaction was run according to the cycle number (N) determined by qPCR. Thermal cycle was as follows:

1 cycle of 98°C for 30 sec

N cycles of 98°C for 10 sec, 63°C for 30 sec, and 72°C for 1 min

Libraries were visualized using gel electrophoresis. 5μL of PCR DNA, 1μL Gel Loading Dye, Orange 6X, and 1μL SYTO60 Red Fluorescent Nucleic Acid Stain 1:1000 in TE Buffer were loaded on a 1.5% Agarose Gel and run in lithium borate buffer at 200v for 20 minutes. The amplified reactions were purified using the QIAGEN MinElute PCR Purification Kit. The libraries were eluted in 10μL nuclease-free water.

Libraries were quantified with qPCR before sequencing. All libraries were sequenced using Nextera single-end sequencing.

ATAC-seq Data Processing and Peak Calling

ATAC-seq reads were aligned to the *Mus Musculus* genome (assembly GRCm38/mm10) using Bowtie2 (version 2.2.2.)[40]. Reads with a MAPQ (mapping quality) score less than 1, that aligned to the mitochondrial genome, or which aligned to unmappable contigs were excluded. Moreover, reads mapping to nuclear mitochondrial sequences (to which reads of mitochondrial

origin may map and contribute to excessively strong signal) were excluded. For visualization of ATAC-seq traces in IgV, base coverage (i.e. number of reads that align to a particular base) was normalized to 1x coverage to control for different total read counts between ATAC-seq libraries. ATAC-seq "peaks", or areas of high ATAC-seq read enrichment that indicate accessible regions of the chromosome, were "called" (i.e. identified) using MACS2[41], which models the baseline rate of read coverage over multiple regions in the genome to calculate the background genome-wide coverage against which peaks are identified.

Enhancer Analysis

We calculated the union of the genomic locations of all peaks across the different replicates and different samples. These "union intervals" showed high concordance with the ENCODE DNase-seq profiles of accessible chromatin (data not shown). Thus, overlapping peaks in FO, MZ, and MZP ATAC-seq tracks would represent accessibility at a common union interval whose genomic range spanned the sequence occupied by any of the peaks. Union intervals that were within 200 bases upstream or 50 bases downstream of a TSS were deemed as part of the promoter in order to reflect the 250-base window that constitutes the core promoter in eukaryotes. TSS annotations of RefSeq-annotated genes were obtained from the UCSC Genome Browser. Non-promoter peaks that were within 10 kb of one another were grouped together to model putative enhancers in the genome. As peaks are non-uniformly spatially distributed across the genome, the 10-kb inter-peak range allowed for spatial coarse-graining of the ATAC-seq landscape. With the counts of reads mapped to enhancers or promoters serving as signals of chromatin accessibility at these regions, we used EBSeq[42] to identify changes in chromatin accessibility at putative enhancers and promoters. EBSeq identified intervals that differed in ATAC-seq read counts across untreated and inhibitor-treated cell types; analyses were carried

out separately for FO, MZ, and MZP B-cells. Differential accessibility of enhancers between conditions was deemed significant if the EBSeq-calculated probability of differential accessibility was 0.95 or greater and if the fold-change in signal was greater than or equal to 2 in either direction.

Motif Enrichment

We used HOMER[43] to detect transcription factor binding sites in enhancers of interest. Binding sites were identified using sequence motifs assembled from ChIP-seq profiles curated from ENCODE consortium data and maintained on JASPAR. We compared motif enrichment between enhancers that decreased in accessibility after inhibitor treatment to enhancers that increased in accessibility after inhibitor treatment. For each motif studied, HOMER performed a binomial test to determine if the relative proportion of peaks in one enhancer set containing the motif was significantly different that the proportion in the other set.

RESULTS

Loss of MZ and MZP B cells following LY-GSI Treatment

Though it has been shown that Notch signaling is required for both MZ and MZP B cell generation, its role post differentiation, is relatively unknown. We sought to further elucidate the role of Notch-signaling in the maintenance of these cells by treating mice with a γ-secretase inhibitor (LY-GSI).

On day 5 following treatment with LY-GSI, there was a significant reduction in both MZ and MZP B cells in the treated mice compared to the PBS-treated (untreated) mice (Figure 3A, B). No significant reduction of FO B cells was observed (data not shown). Within the Live, $CD19^{+}IgM^{hi}IgD^{lo}Cd21^{+}CD1d^{+}$ population, an average of 32.40% of the total cells were MZ B cells in the untreated mice. However, within the same population, only 17.70% of total cells were found to be MZ B cells. A Student's parametric t-test was performed and found that the mean percentage of total cells that were MZ B cells was significantly lower in the LY-GSI treated mice on day 5 following treatment (Figure 3A, $p=0.0155$).

A similar reduction of MZP B cells was also seen in the LY-GSI mice. Within the Live, $CD19^{+}IgM^{hi}IgD^{hi}Cd21^{+}CD1d^{+}$ population, whereas an average of 12.23% of the total cells were found to be MZP B cells in the untreated mice, only 4.55% of total cells within the same population were discovered to be MZP B cells in the LY-GSI treated mice. A Student's parametric t-test determined that the percentage of total cells within the $CD19^{+}IgM^{hi}IgD^{hi}Cd21^{+}CD1d^{+}$ population was significantly reduced in the treated mice 5 days following treatment (Figure 3B, $p=0.0004$).

The reduction in both cell populations therefore demonstrated that Notch-signaling appeared indeed to be required for both mature MZ and MZP B cell maintenance.

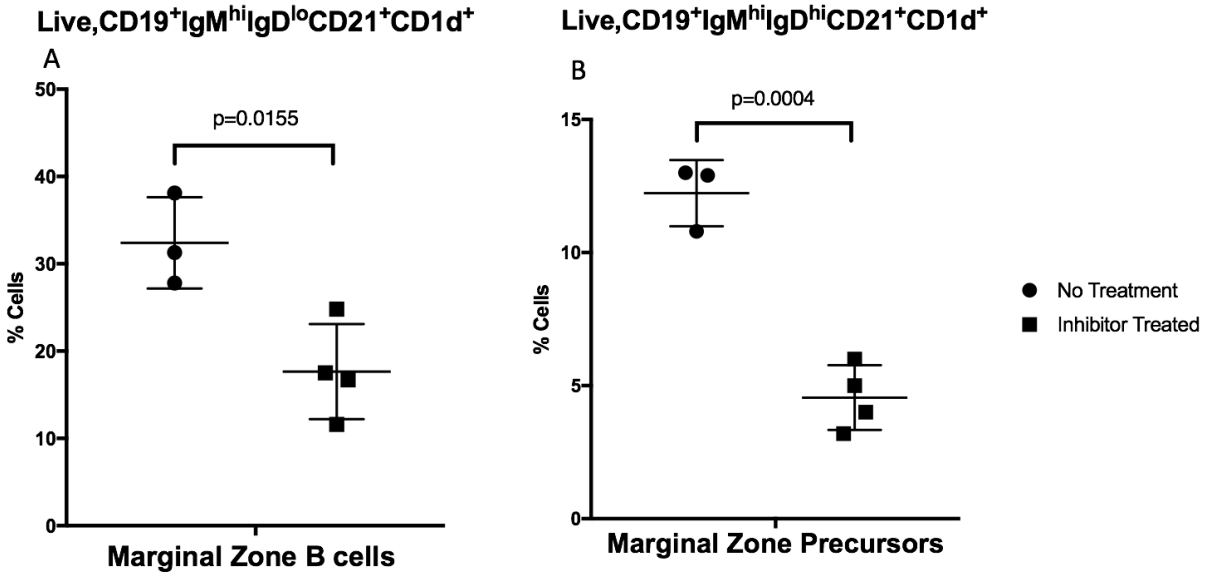

Figure 3. Percentage of MZ and MZP B cells at 5 days post treatment. A) On day 5 following LY-GSI treatment, the percentage MZ B cells in the total Live, $CD19^{+}IgM^{hi}IgD^{lo}Cd21^{+}CD1d^{+}$ population was significantly reduced compared to the percentage of MZ B cells in the untreated mice (mean=17.70% vs. 32.4%, p=0.0155). B) On day 5 following LY-GSI treatment, the percentage MZP B cells in the total Live, $CD19^{+}IgM^{hi}IgD^{hi}Cd21^{+}CD1d^{+}$ population was significantly reduced compared to the percentage of MZP B cells in the untreated mice (mean=17.70% vs. 32.4%, p=0.0155)

GSI-Inhibitor Treatment Led to Varied Accessibility Profiles among Cells

As the LY-GSI treatment appeared to significantly reduce the MZ and MZP B cell populations in the mice spleens, we wanted to further analyze whether this reduction was associated with a chromatin accessibility change.

After mapping ATAC-seq reads to the *Mus Musculus* genome and merging replicates to observe an aggregate of accessibility coverage among replicates, we found that LY-GSI treatment in fact resulted in changes in chromatin accessibility in all cell types studied. While some regions appeared more accessible in the untreated mice, other regions appeared more accessible in LY-GSI treated mice, representing overall changes in the chromatin landscape following inhibitor treatment.

Figure 4 presents an example of a snapshot of the Integrative Genomics Viewer (IGV) for ATAC-seq peaks in specific regions of the genome in MZ B cells of both untreated and LY-GSI treated mice. Peaks representing previously described enhancers were shown under the mapped ATAC signal. On chromosome 7, there appeared to be some regions of the genome that were decreasingly accessible in the surviving MZ B cells of LY-GSI treated mice, as demonstrated by the decreased ATAC signal compared to the untreated mice (Figure 4A). The regions of decreased accessibility shown appeared in promoter regions of the gene Fzd4, a gene encoding the protein Frizzled class receptor 4, a receptor for Wnt protein-ligands. However, on chromosome 11, there appeared to be regions of the genome that were increasingly accessible in MZ B cells of LY-GSI treated mice, as demonstrated by the increased ATAC signal (Figure 4B). For instance, here the regions of increased chromatin accessibility appeared in promoter regions of Sp2, a gene encoding specificity protein 2 (Sp2), a sequence specific transcription factor involved in regulating basic cellular functions[44]. Though these differential accessible peaks are potentially present around genes unrelated to MZ B cell function and although we don't know if these genes are expressed in MZ B cells from both untreated and LY-GSI treated mice, these results demonstrate that indeed Notch-signaling regulates regions of accessible chromatin in MZ

B cells, and by further assessing these regions overall, we'll be able to understand just which biological pathways the Notch receptor is involved in in promoting MZ B cell survival.

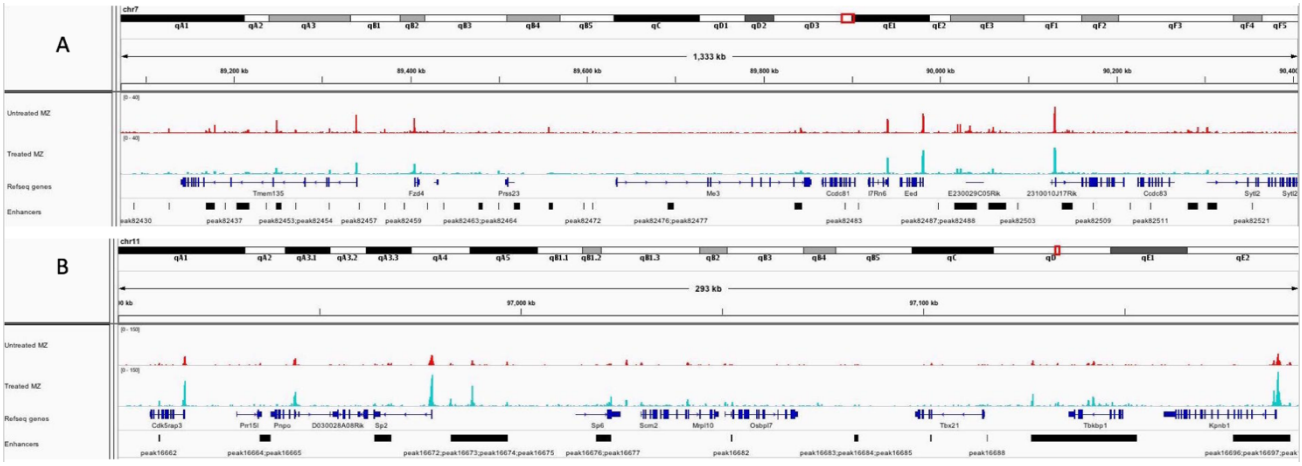

Figure 4. Integrative Genomics Browser snapshot of regions of chromatin in MZ B cells. A) Decreased ATAC signal represents decreased chromatin accessibility in specific regions on chromosome 7 in MZ B cells of LY-GSI treated mice (represented in blue). B) Increased ATAC signal represents regions of increased chromatin accessibility in specific regions on chromosome 11 of LY-GSI treated mice (represented in blue).

Similar instances of regions of chromatin with increasing accessibility and others with decreasing accessibility following LY-GSI treatment were seen in both MZP and FO B cell populations (data not shown). However, in general, as shown in Figure 3, the peaks of differential chromatin accessibility following LY-GSI treatment did not appear to cover large regions of chromatin. Rather than specific regions of chromatin becoming decreasingly accessible following LY-GSI treatment and others becoming increasingly accessible, there were overall systemic changes in chromatin accessibility across all cell types studied.

Thus, following the results of both increased and decreased accessibility in the chromatin of all cell types studied from LY-GSI treated mice demonstrates that Notch-signaling inhibition overall resulted in changes in the chromatin landscape of these cells. It is probable in changing the accessibility landscape of MZ, MZP, and FO cells, LY-GSI treatment altered the regulation

of certain genes as well. However, whether these accessibility changes near certain genes resulted in differential gene expression in the cells following inhibitor treatment will have to be further assessed with whole genome expression analysis in a later study.

Differential Enhancer Accessibility Profiles following GSI-Inhibitor Treatment

Though visualizing merged ATAC-seq reads on IGV gave us a general idea of chromatin accessibility changes along the genome, we wanted to further analyze global chromatin accessibility changes in regulatory regions among cell types after LY-GSI treatment to understand how Notch-signaling inhibition lead to changes in the regulatory landscape of the genome.

Because genomic range of ATAC-seq peaks can show random variation across multiple samples at a particular locus, we calculated the union of genomic locations of all the peaks across the different samples. These "union intervals" showed high concordance with ENCODE DNase-seq profiles of accessible chromatin (data not shown). Thus creating union intervals allowed us to identify regions of the genome where we can compare untreated samples of cells to LY-GSI treated samples to each other. We specifically focused on the non-promoter peaks that were within 10kb of one another that were grouped together to regulatory regions of enhancers in the genome. With the counts of reads mapped to enhancers, EBseq was performed to identify differential accessibility of enhancers across the genome. Differential accessibility of enhancers in cells from different treatment conditions was deemed significant if the EBseq-calculated probability of differential accessibility was 0.95 or greater and if the fold change in accessibility signal was greater or equal to 2 in either direction.

Comparing MZ B cells from untreated mice to MZ B cells from LY-GSI treated mice resulted in the differential accessibility profile of enhancers shown in Figure 5A. The volcano plot demonstrates that there were approximately 183 enhancers that were increasingly accessible in the MZ B cells from untreated mice that were comparatively decreasingly accessible in MZ B cells from LY-GSI treated mice. However, 189 enhancers were also demonstrated to be increasingly accessible in MZ B cells from LY-GSI treated mice that were comparatively decreasingly accessible in the MZ B cells from untreated mice.

Comparing MZP B cells from untreated mice to MZ B cells from LY-GSI treated mice resulted in the differential accessibility profile of enhancers shown in Figure 5B. The volcano plot demonstrates that there were approximately 37 enhancers that were increasingly accessible in MZP B cells from untreated mice. Comparatively, there were approximately 60 enhancers that were increasingly accessible in MZP B cells from LY-GSI treated mice.

Comparing FO B cells from untreated mice to FO B cells from LY-GSI treated mice resulted in differential accessibility profile of enhancers shown in Figure 5C. The volcano plot demonstrates that there were approximately 137 increasingly accessible enhancers in FO B cell populations from untreated mice, while there were approximately 79 increasingly accessible enhancers in FO B cell populations from LY-GSI treated mice.

Notch signaling inhibition significantly affected the regulatory landscape of all cell types following treatment. While some enhancers appeared decreasingly accessible following LY-GSI treatment, there were simultaneously some enhancers that were increasingly accessible. However, as demonstrated in Figure 5, of the many enhancer clusters identified (approximately 10^4), overall, not a large proportion of enhancers experienced accessibility changes following Notch-signaling inhibition. Therefore, it is further demonstrated that Notch-signaling targets very

specific regulatory regions in the genome, some of which may be vital to both MZ and MZP B cell survival upon depleted Notch-signaling.

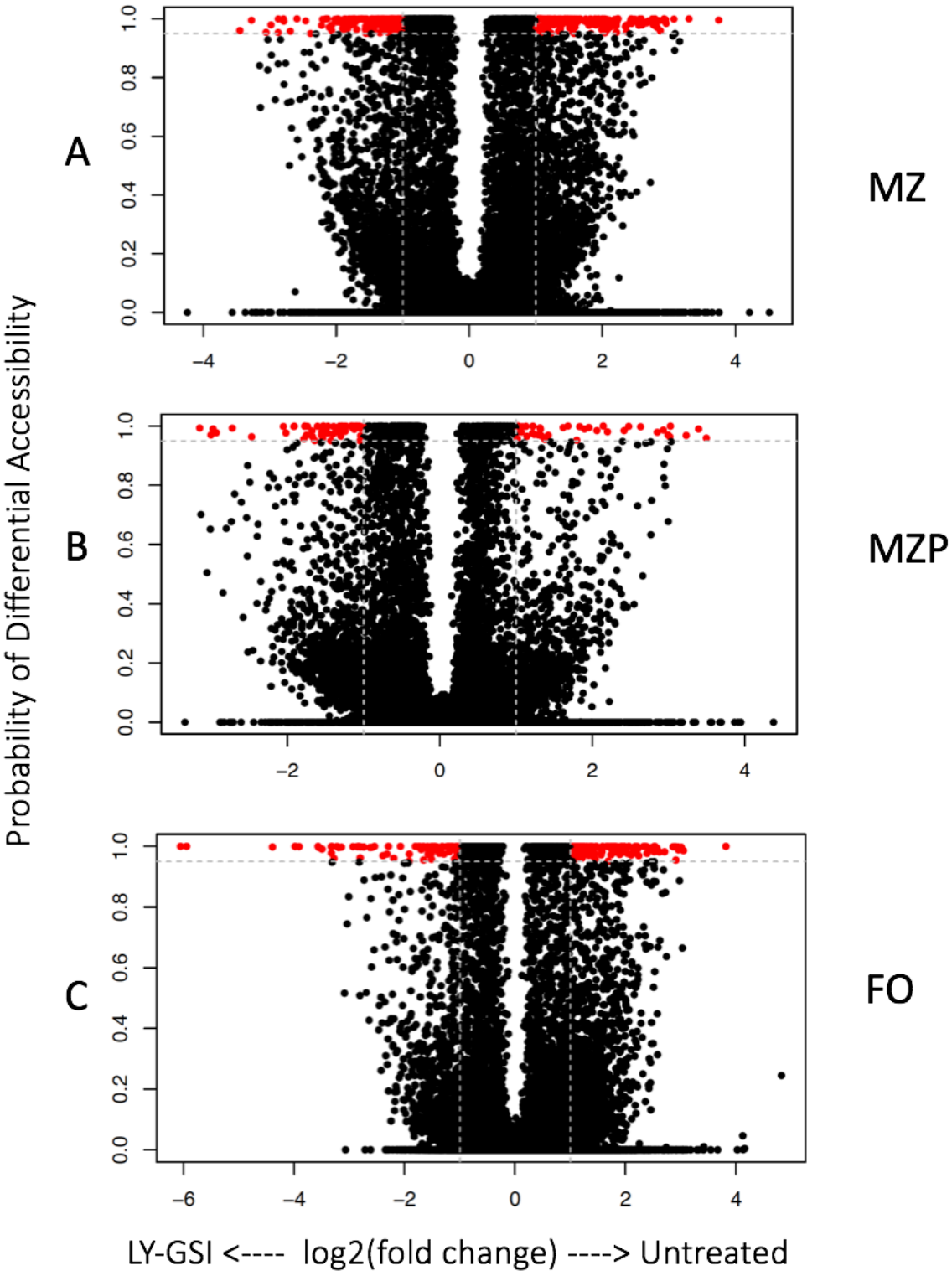

Figure 5. Differential enhancer accessibility. Differential accessibility of enhancers in cells from different treatment conditions was deemed significant if the EBseq-calculated probability of differential accessibility was 0.95 or greater and if the fold change in accessibility signal was

greater or equal to 2 in either direction. Enhancers that were deemed significantly more accessible in cells from one treatment condition are shown in red. A) 183 enhancers were more accessible in MZ B cells from untreated mice, whereas 189 enhancers were more accessible in MZ B cells from LY-GSI treated mice. B) 37 enhancers were more accessible in MZ B cells from untreated mice, whereas 60 enhancers were more accessible in MZ B cells from LY-GSI treated mice. C) 137 enhancers were more accessible in MZ B cells from untreated mice, whereas 79 enhancers were more accessible in MZ B cells from LY-GSI treated mice.

To further understand the profile of the enhancers differentially accessible in WT versus LY treated B cells, we examined the length distributions of enhancers that are affected by Notch inhibition with increased accessibility (Figure 6). In MZ B cells from untreated mice, the median of the distribution of the logarithm of the length of increasingly accessible enhancers was 3.00bp with a standard deviation of 0.62bp. Comparatively, the median of the distribution of the logarithm of the length of increasingly accessible enhancers in MZ B cells from LY-GSI treated mice was 2.51bp with a standard deviation of 0.41bp. A two-sample Kolmogorov-Smirnov (KS) test determined that the distribution of logarithm lengths of enhancers in MZ B cells from untreated mice was significantly different from the distribution of logarithm lengths of enhancers in MZ B cells from LY-GSI treated mice ($D=0.372$, $p=1.07*e^{-11}$). Generally, the lengths of enhancers increasingly accessible in MZ B cells from mice treated with LY-GSI appeared shorter than the lengths of enhancers increasingly accessible in MZ B cells from untreated mice (Figure 6A).

In MZP B cells from untreated mice, the median of the distribution of the logarithm of the length of increasingly accessible enhancers was 2.78bp with a standard deviation of 0.59bp. Comparatively, the median of the distribution of the logarithm of the length of increasingly accessible enhancers in MZP B cells from LY-GSI treated mice was 2.56bp with a standard deviation of 0.33bp. A two-sample KS test similarly determined that the distribution of logarithm

lengths of enhancers in MZP B cells from untreated mice was significantly different from the distribution of logarithm lengths of enhancers in MZP B cells from LY-GSI treated mice (D=0.349, p=0.0076). A trend similar to the one seen for the lengths of enhancers increasingly accessible in MZ B cells from LY-GSI treated mice was observed. The lengths of enhancers increasingly accessible in MZP B cells from mice treated with LY-GSI overall appeared shorter than the lengths of enhancers increasingly accessible in MZ B cells from untreated mice, though there do appear to be a few more outliers of enhancers increasingly accessible of longer lengths (Figure 6B).

In FO B cells from untreated mice, the median of the distribution of the logarithm of the length of increasingly accessible enhancers was 2.57bp with a standard deviation of 0.44bp. Comparatively, the median of the distribution of the logarithm of the length of increasingly accessible enhancers in MZP B cells from LY-GSI treated mice was 2.57bp with a standard deviation of 0.72bp. A two-sample KS test similarly determined that the distribution of logarithm lengths of enhancers in FO B cells from untreated mice was significantly different from the distribution of logarithm lengths of enhancers in FO B cells from LY-GSI treated mice (D=0.313, p=0.00011). However, in FO B cells, the trend of enhancer lengths appeared to differ from the trends observed in MZ and MZP B cells. Generally longer enhancer lengths were increasingly accessible in FO B cells of mice treated with LY-GSI compared to shorter enhancer lengths increasingly accessible in FO B cells from untreated mice, though there do appear to be some outliers representing increasingly accessible enhancers of longer lengths in FO B cells from untreated mice. (Figure 6C).

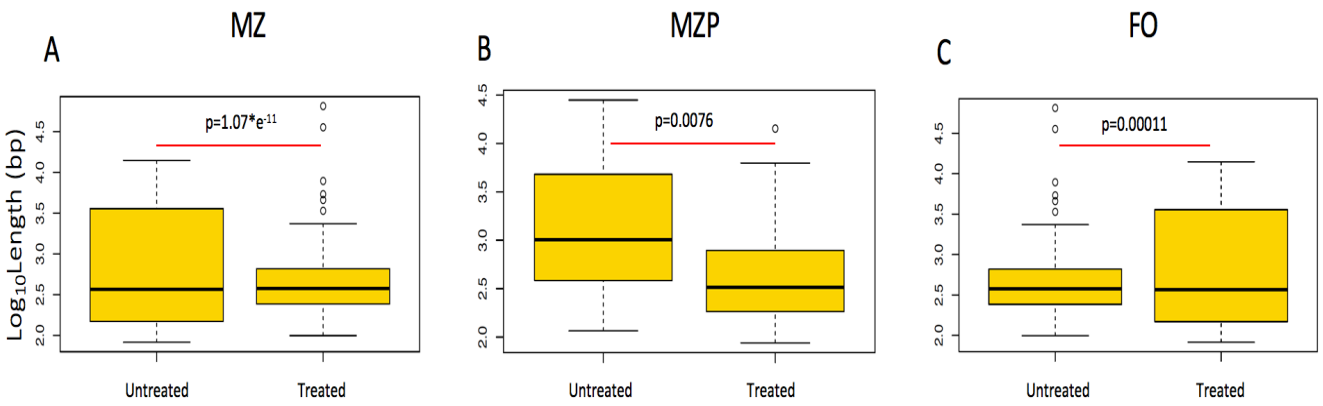

Figure 6. Log_{10}Length distributions of increasingly accessible enhancers. A, B, C) The distribution of Log_{10}Length of enhancers increasingly accessible in all cell types studied from untreated mice were significantly different than the distribution Log_{10}Length of enhancers increasingly accessible in all cell types studied LY-GSI mice (Two-sample KS Test, all $p<0.0076$).

Differential Motif Enrichment in Regions of Increasingly Accessible Enhancers following LY-GSI Treatment

To further understand the enhancer regions both decreased and increased in accessibility in all cell types studied in mice treated with LY-GSI, we used HOMER to detect motifs of transcription binding sites in enhancers clusters of interest, delivering insight into the key molecular players involved in the areas of differentially accessible chromatin. We compared motif enrichment between enhancers that decreased in accessibility following inhibitor treatment (and therefore represented increased accessibility in cell types from untreated mice) and enhancers that increased in accessibility following inhibitor treatment. For each motif studied, HOMER conducted a binomial test to determine if the relative proportion of enrichment of a motif in enhancer set was significantly different from the proportion of enrichment of that motif in the other enhancer set. The motifs significantly enriched in one enhancer set over the other are shown in blue (Figure 7).

As shown in Figure 7, in addition to affecting the chromatin landscape of the cell populations studied, LY-GSI treatment also resulted in differential enrichment of transcription factor motifs in the regions of increasingly accessible chromatin. MZ B cells revealed to be the cell type with the most differential motif enrichment following LY-GSI treatment. Of the motifs differentially enriched in cell types studied, one particularly interesting is the enrichment of CTCF in the surviving MZ B cells of LY-GSI treated mice (Figure 7B). CTCF plays a primary role in regulating the 3D structure of structure of chromatin by linking strands of DNA together forming genome wide loops[45]. CTCF's activity as an insulator is especially intriguing, as insulators are generally found between enhancers and promoters, determining the set of genes enhancers regulate. Thus, CTCF plays an interesting role as a domain barrier, protecting regions

of chromatin from adjacent regions having repressive effects[46]. Therefore, CTCF may play a role in regulating the chromatin landscape of MZ B cells from mice treated with LY-GSI, specifically affecting certain regions of chromatin that become increasingly accessible following treatment.

Though the differential motif enrichment of B cells from both untreated and LY-GSI treated mice doesn't provide us direct information about the potentially distinct gene expression patterns in the different cell types studied, it further demonstrates that not only does Notch-signaling indeed have an effect on shaping the regulatory landscape of the B cells studied, but it also has a potential role in affecting the transcription factors available in those accessible regions. Because these transcription factors may be related to genes potentially differentially expressed and responsible for cell-specific functions, the motif enrichment analysis provides a foundation of gene-regulating targets that may reveal importance for both MZ and MZP B cell survival.

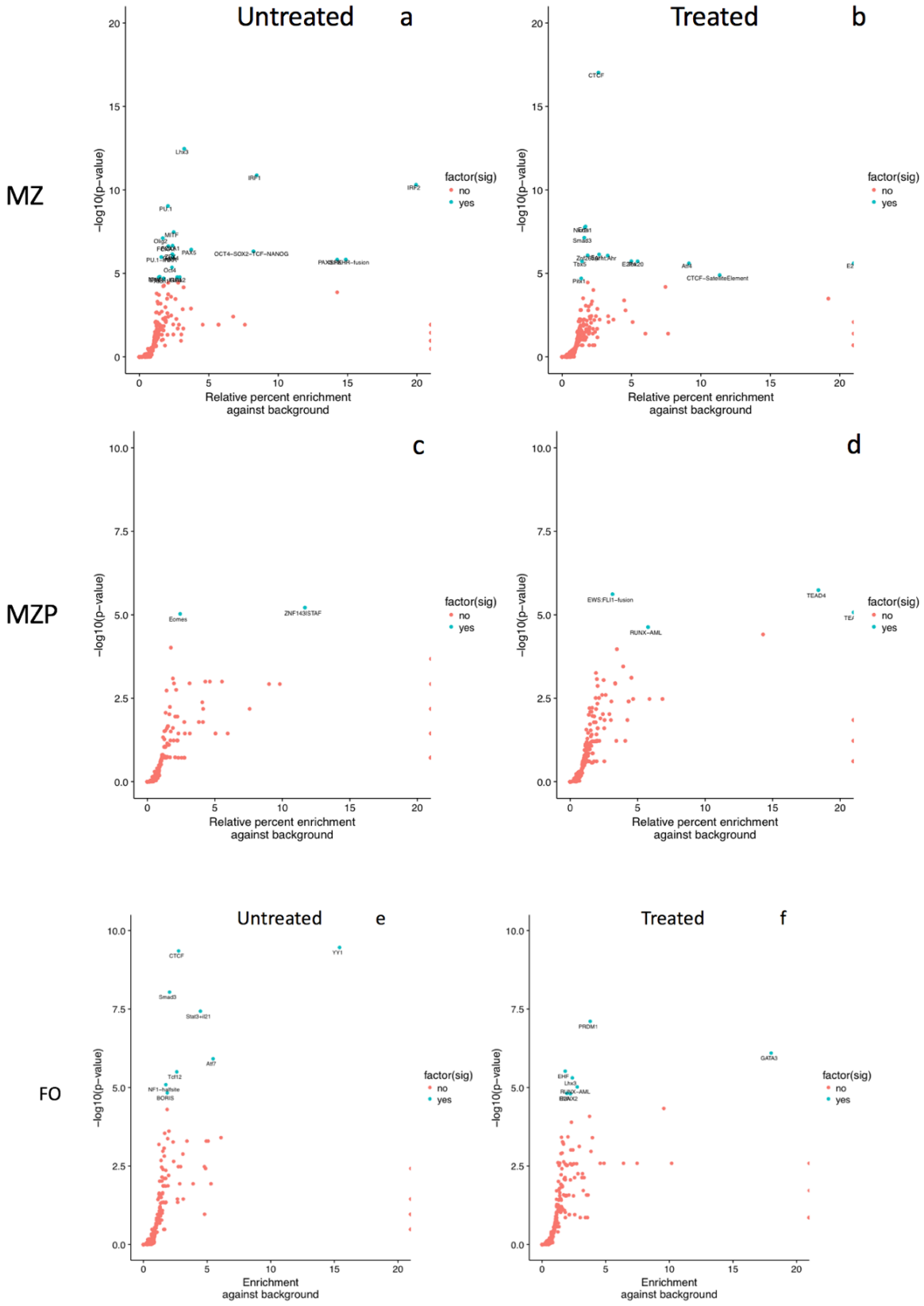
MZ
MZP
FO
Untreated
a
Treated
b
c
d
Untreated
e
Treated
f
−log10(p−value)
Relative percent enrichment against background
Enrichment against background
factor(sig)
no
yes
CTCF
IRF1
IRF2
PU.1
OCT4−SOX2−TCF−NANOG
CTCF−SatelliteElement
Eomes
ZNF143/STAF
EWS:FLI1−fusion
RUNX−AML
TEAD4
YY1
Smad3
Stat3+H21
Atf7
Tcf12
NF1−Halfsite
BORIS
PRDM1
GATA3
EHF
Lhx3

Figure 7. Differential motif enrichment in regions of increasingly accessible enhancers. Motifs that demonstrated significantly different enrichment in accessible enhancer regions from HOMER analysis are shown in blue.

DISCUSSION

Notch Signaling is Required for MZ and MZP B Cell Maintenance in the Spleen

Though it has been shown that Notch signaling is required for both MZ and MZP B cell generation[5], its role post differentiation, is relatively unknown. We sought to further elucidate the role of Notch-signaling in these cells post differentiation by treating mice with a γ-secretase inhibitor. γ-secretase is required for Notch intracellular domain (NICD) cleavage, resulting translocation to the nucleus, and interaction with various DNA binding proteins to assemble a transcription complex that will activate downstream genes. Specifically, the Notch2-signaling pathway has been implicated as a requirement for marginal zone B cell fate commitment through the interaction of NICD with the DNA binding protein RBJ-κ to form a transcription complex with the coactivator MAML1. A recent study found that treating adult mice with an antibody blocking DL-1, a known ligand for Notch-signaling, depleted pre-established MZ B cell populations in adult mice[17], implicating a role of Notch2-signaling in the maintenance of MZ B cells in the spleen.

After treating mice with intact spleens with a γ-secretase inhibitor, on day 5 we observed a significant loss of both MZ and MZP B cells compared to the population of those cells in the untreated mice. As MZ B cells are long-lived and self-renewing, it is unlikely that the many precursors to MZ and MZP B cells (i.e. T2 B cells) were differentiating into either MZ or MZP B cells during this time frame. Therefore, in an environment restricted from Notch-signaling, many MZ and MZP B cells were unable to survive. Thus, the reduction in cell numbers demonstrates that in addition to playing a role in MZ B cell fate commitment, Notch-signaling plays a role in

regulating the maintenance and enhancing the survival of both MZ B and MZP B cell populations.

Notch-Signaling Perturbation affects the Chromatin Landscape in Surviving B Cells

Using ATAC-seq we were able to harness the potential of a relatively novel field of biotechnology to visualize the changes in regions of chromatin accessibility following a depletion of Notch-signaling.

Our results assessing the chromatin landscape in MZ, MZP, and FO B cells following Notch-signaling perturbation are rather interesting. Following a depletion of Notch-signaling, not only do differential regions of chromatin become less accessible, but others become increasingly accessible. What is more interesting is that this finding of differential accessibility was found in all cell types studied, not just MZ and MZP cells where we expected with a loss of populations numbers that there may be a significant regulatory change in the surviving cells. Though Notch has been reported to be invaluable to both MZ and MZP B cell development, it is not required for the survival of FO B cells, as enhanced BCR signaling characteristic of driving the FO B cell fate often inhibits Notch signal transduction during FO B cell development[5]. However, according to Immgen, FO B cells do express Notch2 on their surface, and here we show that though Notch-signaling may not be invaluable to FO B cell survival, it is affecting the chromatin accessibility and the regulatory domain of this cell type, opening a door to a previously unstudied area of research. Because Notch-signaling is not required for FO B cell development, we did not expect Notch-perturbation to significantly affect FO B cells. Yet, given that there were indeed changes in the regions of accessible chromatin, begs the question, what is Notch regulating in FO B cells, and how are those regions, if at all, important for FO B cell function and identity?

On the order of magnitude of enhancer clusters studied (approximately 10^4) Notch-signaling perturbation did not in fact lead to vast regions of differential chromatin accessibility. Instead, it appeared to affect relatively small regions of enhancer clusters, some which, following a depletion of Notch-signaling, were decreasingly accessible, and others which were increasingly accessible. This differential accessibility in all cell types studied indicates that Notch signal transduction is affecting the accessibility of increasingly specific regions of chromatin, where it may play a key role, especially in the case of MZ and MZP B cells, in regulating maintenance and survival. This finding of small subset of specific regulatory regions affected by Notch perturbation is further supported by research carried out by Wang, et al. (2013) who investigated regulatory regions termed "dynamic Notch sites[47]." These researchers identified a subset of Notch-binding sites, approximately only 10% of all Notch sites in the genome, that change in occupancy when Notch-signaling is inhibited. Furthermore, they discovered that these "dynamic Notch sites" are in key regulatory regions of the genome containing giant regulatory switches, stretch enhancers, known to be vitally important in regulating core genes for cell specificity and function.

The discovery that "dynamic Notch sites" are located in regions of large core enhancers is especially interesting given our finding about the length of enhancers affected following Notch-perturbation. In both MZ and MZP B cells, the length of enhancers that became decreasingly accessible following LY-GSI treatment were overall, significantly longer than the length on enhancers that became increasing accessible after treatment. Given that stretch enhancers are clusters of longer enhancer regions, we can infer that the regions of enhancers that became decreasingly accessible following Notch perturbation may have been involved in core regulatory functions characteristic of stretch enhancers important for cell function and survival.

Thus, these regions of stretch enhancers may provide insight into Notch-regulated events that enhanced the survival of both MZ and MZP B cells, of which without, many cells were unable to survive. Of equal interest, however, is the regions of small enhancer clusters that became increasingly accessible in both MZ and MZP B cells after Notch-inhibition. As the cells we collected were those that survived Notch-perturbation, these regions of smaller enhancers may provide insight into regulatory mechanisms that rescued surviving MZ and MZP B cells. As indicated by their surface receptors, these cells collected from a Notch-depleted environment are still of MZ and MZP B cell identity, and thus, the specifics of these regions of enhancers and genes regulated and expressed by them are an interesting field for further research.

Differential Chromatin Accessibility following Notch-signaling Perturbation gives Insight into Key Molecular Players involved in Managing the Regulatory Landscape of MZ B cells

With Motif enrichment analysis, we were able to determine that not only does Notch-signaling perturbation result in differential regions of accessible chromatin in the cell types studied, but the transcription factor motifs enriched in these differential regions of chromatin accessible are distinct as well. This finding further supports that Notch-signaling is involved in distinct regulatory mechanisms that change when its signal is depleted. Though as of now we don't know which genes are differentially expressed following Notch-perturbation, motif enrichment analysis gives us insight into molecular players that may be involved in distinct biological pathways for enhancing the survival of particularly MZ and MZP B cells. Further investigation into the genes surrounding these enriched transcription motifs may also provide an interesting insight into their regulatory function.

One finding from the motif enrichment analysis of particular interest is the enrichment of CTCF in MZ B cells following Notch-perturbation. CTCF plays an intriguing role as an insulator, preventing interactions between adjacent regions of the genome that are not conducive for cell function[44]. Interestingly, in their study of "dynamic Notch sites," Wang, et al. (2013) found that Notch regions of particular importance for regulating core cell functions often coincide spatially with CTCF-binding sites. Thus, the enrichment of CTCF in surviving MZ B cells from LY-GSI treated mice indicates that CTCF may play a role in regulating survival signals in the absence of Notch-signaling in MZ B cells. A possible mechanism for this potential rescuing effect of CTCF may be through its interaction with RBP-Jκ, the DNA binding protein significantly enriched in "dynamic Notch sites." An investigation conducted by Lake, et al. (2014) revealed that genomic sites enriched for RBPJ occupancy are simultaneously enriched for CTCF and that the two molecular players interact[48]. In the absence of Notch-signaling, RBP-Jκ often exerts repressive effects on Notch-target genes[49]. Thus, one possible mechanism for the role of enriched CTCF in surviving LY-GSI treated MZ B cells is acting as an insulator for the repressive effects of RBP-Jκ on adjacent regions of the genome. This insulation of RBP-Jκ by CTCF may play an intriguing role in maintaining regulatory mechanisms important for MZ B cell survival, particularly in the absence of Notch-signaling, and provides an intriguing avenue for further research.

Future Directions

The work presented here provides incredible insight into the relatively unknown effects of Notch-signaling in enhancing the maintenance and survival of MZ and MZP B cells by affecting the chromatin accessibility landscape of these cell populations. However, additional work needs to be conducted to determine the cellular mechanisms through which Notch-signaling is providing potentially survival signals to MZ and MZP B cells.

One avenue through which to further investigate specific function of Notch-signaling in MZ and MZP cells is to analyze the genes commonly associated with the regions of chromatin that become decreasingly accessible following Notch-perturbation. These genes may be involved in regulating core functions important for the survival of MZ and MZP B cells, and understanding the pathways they are involved in would further elucidate the mechanism through which Notch-signaling is affecting MZ and MZP B cell maintenance. In addition to this analysis, it is essential to conduct RNA-seq on the FO, MZ, and MZP B cells from both the untreated and LY-GSI treated mice. RNA-seq would provide invaluable insight into the differential expression of genes in cells before and after treatment, which would further elucidate the mechanisms through which Notch-signaling is affecting gene expression specifically, and not just the regulatory chromatin landscape. During this study, we additionally collected cells from both untreated and LY-GSI treated mice to conduct RNA-seq, therefore it will be of utmost priority in the months to conduct this additional analysis.

In addition to further investigating the role of Notch-signaling in MZ and MZP B cell survival, an interesting direction of research would be to assess the role of Notch-signaling in Follicular B cells, as the research presented here indicates that Notch does affect the regions of accessible chromatin in these cells. By comparing the regions of accessible chromatin in MZ,

MZP, and FO B cells, we may be able to find regulatory regions that are cell-specific. If these regions are common among all cell types, it would indicate that the Notch-receptor is providing common signals across the different cell populations. However, if these regions of accessible chromatin are different, we would be further able to differentiate between the regulatory elements specific to MZ and MZP B cell survival provided by the Notch-receptor, and potentially novel pathways particular to FO B cells.

In conclusion, the work presented here provides evidence for a novel role of the Notch-receptor in providing survival signals to mature MZ and MZP B cell populations. In addition, Notch-signaling is involved in shaping the regulatory landscape of accessible chromatin in MZ, MZP, and FO B cells. Specifically, in MZ and MZP B cells, the signals provided by the Notch receptor are indicated to be involved in maintaining regulatory regions of stretch enhancers, which are known to be involved in core functions vital to cell-specific survival and function. Further research can potentially determine the mechanism through Notch-signaling is promoting the survival of mature MZ and MZP B cells and is of incredible interest to complete this story.

ACKNOWLEDGEMENTS

The work presented here would not be possible without vital contributions from my numerous mentors at the Ragon Institute. I would like to thank the Outreach, Education, and Training program and Sylvie Le Gall for allowing me to cultivate my research skills last summer, Vinay Viswanadham for his vast computational knowledge and help with the chromatin accessibility profiling, and finally, Vinay Mahajan and Shiv Pillai for their unwavering support and mentorship throughout this journey.

SUPPLEMENTARY DATA

Supplementary Table 1. Oligo primers. A list of the ATAC-seq oligos used for PCR.

Ad1_noMX:	AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTCAGATGTG
Ad2.1_TAAGGCGA	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGT
Ad2.2_CGTACTAG	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGT
Ad2.3_AGGCAGAA	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGT
Ad2.4_TCCTGAGC	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGT
Ad2.5_GGACTCCT	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGT
Ad2.6_TAGGCATG	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGT
Ad2.7_CTCTCTAC	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGT
Ad2.8_CAGAGAGG	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGGAGATGT
Ad2.9_GCTACGCT	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTCTCGTGGGCTCGGAGATGT
Ad2.10_CGAGGCTG	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGGAGATGT
Ad2.11_AAGAGGCA	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGGAGATGT
Ad2.12_GTAGAGGA	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGGAGATGT
Ad2.13_GTCGTGAT	CAAGCAGAAGACGGCATACGAGATATCACGACGTCTCGTGGGCTCGGAGATGT
Ad2.14_ACCACTGT	CAAGCAGAAGACGGCATACGAGATACAGTGGTGTCTCGTGGGCTCGGAGATGT
Ad2.15_TGGATCTG	CAAGCAGAAGACGGCATACGAGATCAGATCCAGTCTCGTGGGCTCGGAGATGT
Ad2.16_CCGTTTGT	CAAGCAGAAGACGGCATACGAGATACAAACGGGTCTCGTGGGCTCGGAGATGT
Ad2.17_TGCTGGGT	CAAGCAGAAGACGGCATACGAGATACCCAGCAGTCTCGTGGGCTCGGAGATGT
Ad2.18_GAGGGGTT	CAAGCAGAAGACGGCATACGAGATAACCCCTCGTCTCGTGGGCTCGGAGATGT
Ad2.19_AGGTTGGG	CAAGCAGAAGACGGCATACGAGATCCCAACCTGTCTCGTGGGCTCGGAGATGT
Ad2.20_GTGTGGTG	CAAGCAGAAGACGGCATACGAGATCACCACACGTCTCGTGGGCTCGGAGATGT

Ad2.21_TGGGTTTC	CAAGCAGAAGACGGCATACGAGATGAAACCCAGTCTCGTGGGCTCGGAGATGT
Ad2.22_TGGTCACA	CAAGCAGAAGACGGCATACGAGATTGTGACCAGTCTCGTGGGCTCGGAGATGT
Ad2.23_TTGACCCT	CAAGCAGAAGACGGCATACGAGATAGGGTCAAGTCTCGTGGGCTCGGAGATGT
Ad2.24_CCACTCCT	CAAGCAGAAGACGGCATACGAGATAGGAGTGGGTCTCGTGGGCTCGGAGATGT

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