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LSD1 regulates marginal zone B cell development, B cell proliferation, and plasmablast differentiation

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LSD1 regulates marginal zone B cell development, B cell proliferation, and plasmablast differentiation

by

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B.S., University of Illinois at Urbana-Champaign, 2014

Advisor: Jeremy M. Boss

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Abstract

LSD1 is a histone demethylase that primarily targets H3K4me1/2 and H3K9me1/2, leading to transcriptional repression and activation, respectively. Through its demethylase function, it promotes cellular processes such as autophagy, cell cycle progression, and inflammation, and drives the development and differentiation of multiple cell types including adipocytes, embryonic stem cells, blood cells, myocytes, neurons, and gametocytes. Additionally, LSD1 has been shown to contribute to human diseases such as cancer and viral infection. Despite the extensive research on the role of LSD1 throughout normal and disease pathways, much remains to be discovered. This thesis will focus on the role of LSD1 in B cell development and differentiation into antibody-secreting plasma cells. A 2009 study showed that LSD1 directly interacted with the key plasma cell transcription factor Blimp-1, suggesting an *in vivo* role for LSD1 during B cell differentiation. Here I show that B cell-conditional deletion of LSD1 in mice results in diminished B cell proliferation and differentiation in response to the antigen lipopolysaccharide (LPS). Genome-wide transcriptome and chromatin accessibility analyses showed that LSD1 repressed hundreds of genes in LPS-induced plasma cells. These genes were in close proximity to binding sites of the key B cell differentiation transcription factors Blimp-1, IRF4, and PU.1. LSD1 suppressed chromatin accessibility and H3K4me1 at these target binding sites, implying that LSD1 directly regulates multiple transcription factor networks throughout B cell differentiation. Quantification of developing B cell populations revealed that LSD1-deficient bone marrow B cell development is normal, but marginal zone B cell development in the spleen is impaired. Similar to its role in B cell differentiation, LSD1 repressed hundreds of genes in marginal

zone B cells. Chromatin accessibility analysis showed that LSD1 repressed accessibility at the binding sites of transcription factors involved in splenic B cell development, including NF-κB. *In vitro* marginal zone B cell development experiments solidified a key role for LSD1 in regulating non-canonical NF-κB signaling induced by BAFF. Indeed, LSD1 directly interacted with the non-canonical NF-κB transcription factor p52. Overall, these studies not only define LSD1 as a critical epigenetic and transcriptional regulator during B cell development and differentiation, but also provide novel mechanistic insights into how LSD1 regulates these processes. The revealing of this new B cell branch of LSD1 function will be critical to understanding how epigenetic modifying proteins contribute to B cell-based diseases and may give rise to innovative treatment options for such diseases.

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Chapter 1: Introduction to LSD1

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Chapter 1 Part A: The role of LSD1 throughout cellular development, differentiation, function, and disease

Rationale

The thesis presented below covers the multiple known biological roles of LSD1 (chapter 1), the role of LSD1 in B cell proliferation and differentiation (chapter 2), and the role of LSD1 in marginal zone B cell development (chapter 3). My experimental work, which fleshes out molecular mechanisms by which LSD1 regulates B cell development and differentiation (chapters 2 and 3), will be critical to further understand the epigenetic regulation of the humoral immune response. In general, the humoral immune response is mediated by antibody-secreting cells derived from B cell differentiation in response to antigen. The humoral immune response is relevant to public health in that 1) the generation of antibody-secreting cells through vaccines induces population-level immunity against dangerous infectious diseases, 2) B cell dysregulation can result in autoimmunity and immunodeficiency, and 3) B cell-based cancers, such as leukemia and multiple myeloma, result in thousands of deaths per year[1,2]. By understanding the epigenetic regulation of B cell development and differentiation, insights into novel vaccine methodologies and novel treatments for B cell-based diseases can be gained. Given the vast array of known disease treatments targeting LSD1[3,4], studying the role of LSD1 in developmental and differentiation pathways known to give rise to diseases, such as B cell development and differentiation, should be prioritized.

Biochemical and Functional Characterization of LSD1

Post-translational histone modifications are a dynamic network that epigenetically regulate gene expression[5,6]. Histone methylation is integral to reinforce cellular identity, facilitate responses to extracellular signals, and drive developmental and differentiation pathways[7-9]. Histone methyltransferases are responsible for actively writing histone methylation marks, the first one identified being SUV39H1 by Rea et al. in 2000[10]. Shortly after in 2004, Shi et al. identified the first histone demethylase as LSD1, proving that histone methylation is a dynamic modification[11]. Since then, numerous studies have shown dozens of enzymes to be responsible for writing and erasing the histone methylation[8,9]. Although the enzymatic function of many histone modifying enzymes has been well characterized, the diverse *in vivo* functional roles of each enzyme are only starting to be revealed. Given its well-defined function and its ubiquitous expression across multiple cell types, LSD1 has and continues to represent a prime candidate for continued *in vivo* functional studies.

Lysine-specific demethylase 1 (LSD1), also known as lysine demethylase 1A (KDM1A), amine oxidase flavin-containing domain 2 (AOF2), and BRAF35-HDAC Complex Protein 110 (BHC110), is a highly conserved histone demethylase present in all major eukaryotic lineages[11]. LSD1 consists three protein domains: an N-terminal Swi3, Rsc8, and Moira (SWIRM) domain, a coiled coil tower domain, and a C-terminal amine oxidase (AO) domain. The AO domain demethylates lysine residues through a FAD-dependent amine oxidation reaction[12,13]. Specifically, catalysis utilizes FAD and molecular oxygen, involves the formation of an imine intermediate, and generates hydrogen peroxide and formaldehyde. The SWIRM domain forms a hydrophobic interface with the AO domain, some of which is necessary for catalytic function[14]. Both the SWIRM domain and

the tower domain facilitate protein-protein interactions that promote corepressor and coactivator complex formation[12,14].

Through its enzymatic function, LSD1 operates as a transcriptional rheostat. LSD1 demethylates histone 3 lysine 4 mono- and di-methylation (H3K4me1/2)[11] and histone 3 lysine 9 mono- and di-methylation (H3K9me1/2)[15] to repress or activate gene transcription, respectively (**Fig. 1-1 A, B**). In one case, LSD1 has also been shown to demethylate histone 4 lysine 20 methylation monomethylation (H4K20me1) in gene bodies to activate transcription[16] (**Fig. 1-1C**). In addition to demethylating histones, LSD1 has been shown to directly demethylate proteins to influence their stability and function, including p53[17], E2F1[18], DNMT1[19], HIF1α[20], MYPT1[21], MEF2D[22], STAT3[23], p65[24], Tat[25], and IFITM3[26]. LSD1 shares the LSD/KDM1 family with its homologue LSD2, which is also capable of demethylating H3K4me1/2 and regulating transcription[3].

Prior to knowledge of its identity as a histone demethylase, LSD1 was found to be part of corepressor complexes containing histone deacetylases in HeLa cells, including the CoREST complex that represses neuronal genes in non-neuronal cells[27-31]. Following its identification as a histone demethylase in 2004[11], its properties were further elucidated. By 2006, the crystal structure of LSD1 had been deciphered, solidifying its identity as a monoamine oxidase and providing insight into its interactions with target histone substrates and CoREST[32,33]. Additional experiments characterized reaction kinetics, as well as confirmed the ability of histone modifications within the 21 N-terminal amino acids of H3 to modulate LSD1 enzymatic function[34-36]. Specifically, LSD1 preferentially targets nucleosomes lacking histone modifications such as H3K9ac and H3S10 phosphorylation. These experiments pointed towards a sequential corepressor complex-based model of gene

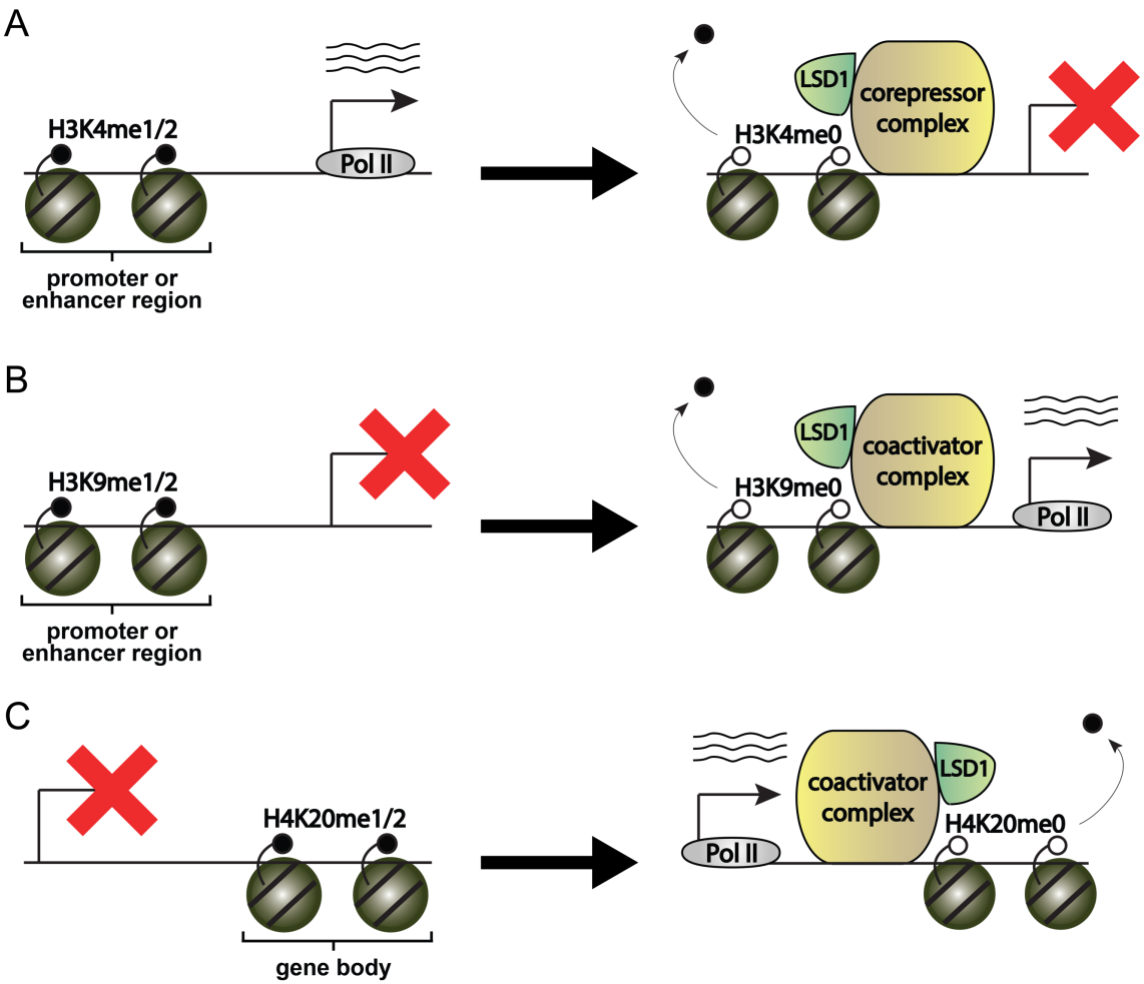


Figure 1-1 – The histone demethylase functions of LSD1
LSD1-mediated demethylation of **(A)** H3K4me1/2, **(B)** H3K9me1/2, and **(C)** H4K20me1/2.

repression: 1) HDAC activity deacetylates target chromatin; 2) LSD1 recognizes deacetylated histones on target chromatin and demethylates H3K4me1/2.

The interaction of LSD1 with protein complexes is required for its function through recruiting LSD1 to target genomic loci. This was first established through experiments examining the relationship between LSD1 and CoREST, showing that CoREST interaction is necessary for LSD1-based H3K4 demethylation and gene repression by bridging LSD1 to nucleosomal susbtrates[36,37]. Interaction with CoREST was also shown to protect LSD1

from proteasomal degradation[36]. In 2005, it was shown that LSD1 can directly interact with androgen receptor (AR) to stimulate AR-dependent transcription via demethylation of H3K9me1/2 repressive marks[15]. Purified LSD1 was capable of demethylating H3K4 but not H3K9[11], and one mechanism for this activation function was recently identified by a 2015 study showing that an isoform of LSD1, LSD1+8a, can demethylate H3K9 due to a modified substrate binding cleft of the AO domain[38,39]. Additional studies also suggest that LSD1-mediated H3K9me demethylation does not depend on the proteins it forms complexes with, but instead is influenced by resident histone modifications, such as phosphorylated H3T11, which promotes H3K9me demethylation[40], and phosphorylated H3T6, which suppresses H3K4me demethylation[41].

These important studies extensively characterized the properties of LSD1 and provided a foundation to study its *in vivo* role throughout developmental and disease pathways, which is the subject of Chapter 1 Part A. Here I organize and discuss the role of LSD1 in normal key molecular processes and developmental pathways (**Fig. 1-2 A**). Second, I examine the role of LSD1 in promoting, maintaining, or protecting against disease pathologies (**Fig. 1-2 B**). Finally, I explore the different methods of pharmacologically targeting LSD1 for disease treatment and current LSD1-based clinical trials.

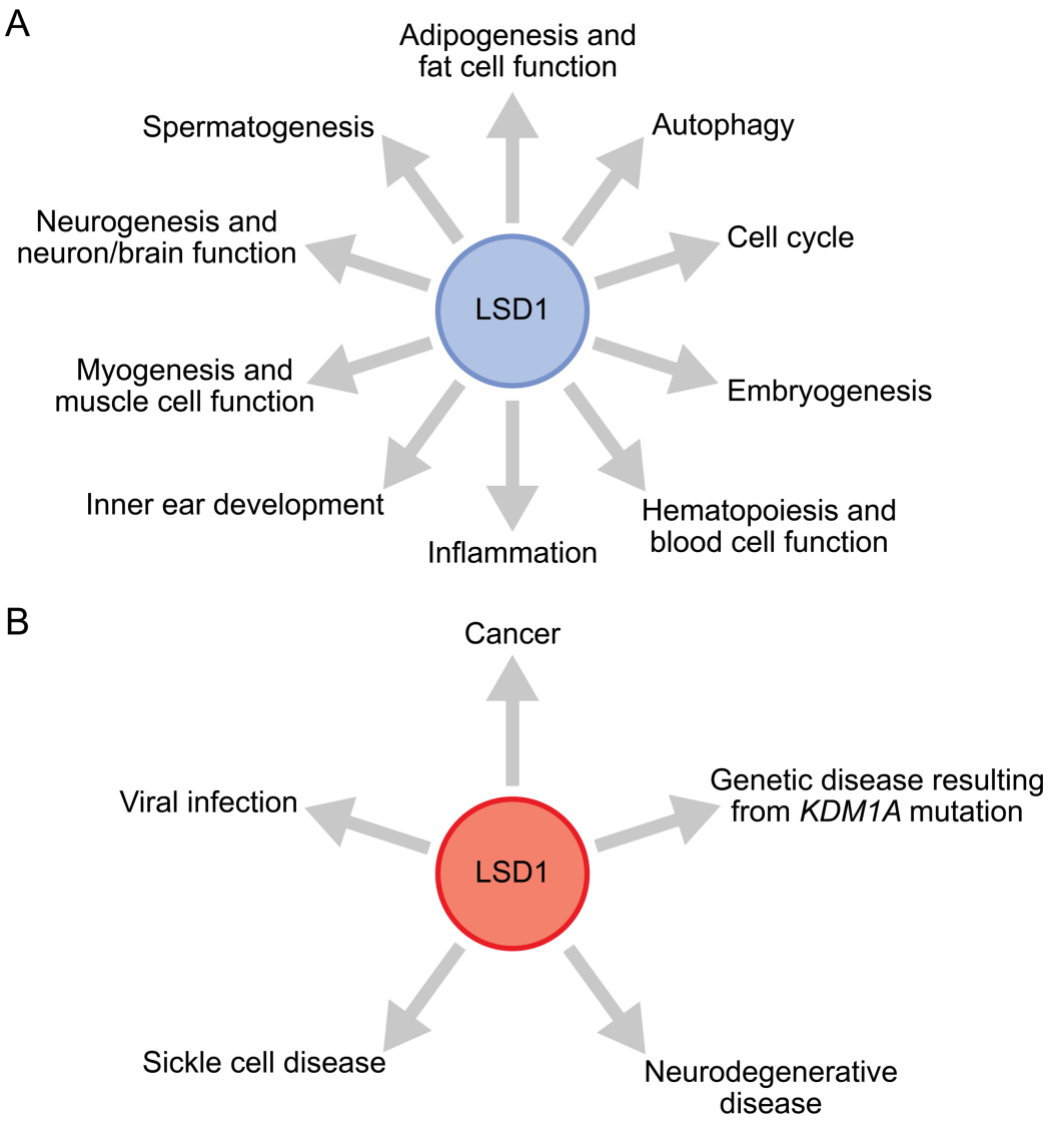


Figure 1-2 – The roles of LSD1
(A) The normal cellular developmental/differentiation pathways and functions that LSD1 regulates. **(B)** The diseases in which LSD1 plays a role in.

Cellular Development, Differentiation, and Function

Adipogenesis and Fat Cell Function

Adipogenesis is the differentiation of pre-adipocytes into white or brown adipocytes and is highly regulated by multiple signaling cascades, downstream of which the key transcription factors C/EBPα and PPARγ function to promote adipocyte cell fate commitment[42]. A critical role for H3K4 methylation during adipogenesis was first identified in 2006 when Musri *et al*. showed that genes essential for adipogenesis, including *Adipoq* (encodes adiponectin), exhibited H3K4 methylation at their promoter, and that this methylation was necessary for *in vitro* adipogenesis from 3T3-L1 fibroblasts[43]. This role was further highlighted in 2008 when Lee *et al*. showed that MLL3, an H3K4me1/2 methyltransferase, is necessary for *in vivo* white fat formation, response to inducers of adipogenesis, and induction of the PPARγ-target gene *Fabp4*[44]. It was later revealed in 2010 that LSD1 is both induced during and necessary for *in vitro* adipogenesis, functioning to demethylate H3K9me2 at the promoters of *Cebpa* and *Pparg* to induce their expression[45,46]. LSD1 has also been shown to suppress adipogenic differentiation from the human ESC line H9, possibly through H3K4me2 demethylation-mediated repression of target genes, such as *Cebpa* and *Pparg*[47].

Additional studies have identified a more intricate role for LSD1 during and following *in vivo* adipogenesis. Conditional knockout experiments in mice have shown that LSD1 is necessary for the differentiation of white adipocytes[48], which are responsible for storing energy in the form of lipids. Furthermore, LSD1 is essential for early versus late adipocyte differentiation, as adipocytes form normally even if LSD1 is deleted after

three days following preadipocyte stimulation[48]. Generation of brown adipocytes, which are mitochondria-dense adipocytes that regulates energy homeostasis by producing heat without ATP generation, is also regulated by LSD1[49]. Specifically, LSD1 functions to repress the Wnt signaling pathway through H3K4 demethylation at Wnt gene promoters, which promotes the differentiation of brown adipocytes from preadipocytes.

Regarding adipocyte function, LSD1 was first shown in 3T3-L1-derived adipocytes to maintain repression of genes involved in energy expenditure and oxidative metabolism, such as PPARγ coactivator-1α, via H3K4 demethylation[50]. LSD1 is in fact upregulated in white adipose tissue upon cold or nutritional imbalance stimuli, which leads to an increase in oxidative metabolism gene expression[48]. Mechanistically, LSD1 cooperates with the transcription factor NRF1 to facilitate this effect, and LSD1 overexpression in white adipocytes results in the induced expression of oxidative metabolism genes. LSD1 is also responsible for maintaining beige adipocyte identity by promoting the expression of PPARα, as age-programed depletion of LSD1 drives the transition of beige adipocytes into white adipocytes through a PPARα-repressed mechanism[51]. LSD1 further maintains beige adipocyte identity by interacting with PRDM16 to repress white fat-selective genes via H3K4me1/2 demethylation, but also promotes brown adipocyte thermogenesis through non-PRDM16-based repression of HSD11B1, a glucocorticoid-activating enzyme[52]. Overall, these studies show that LSD1 is a critical regulator of adipocyte formation and function, and suggest that it is necessary for their ability to maintain energy homeostasis and survival under strenuous conditions.

Autophagy

Autophagy is an essential cellular process that consists of the degradation of cytoplasmic components such as damaged organelles and protein aggregates within lysosomes, leading to molecular recycling[53]. Under homeostatic conditions, autophagy occurs at low basal levels, however it is rapidly upregulated when cells are exposed to certain conditions such as nutrient starvation or have increased bioenergetic needs such as during differentiation[53]. Multiple factors function to repress autophagy under homeostatic conditions, including mTOR signaling, because aberrant upregulation can cause cellular defects and apoptosis[53]. A clear role for LSD1 in repressing autophagy was identified in 2015 when Periz et al. showed that in HEK293 cells, LSD1 directly demethylated p53 to suppress its transcriptional activation of the proteasomal degradation and autophagy pathways[54]. Studies in multiple cancer cell lines revealed additional mechanisms by which LSD1 repressed autophagy. In mouse hepatocytes, treatment with the late fed-state hormone FGF19 activates the transcription factor SHP, which recruits LSD1 to target autophagy genes such as *Tfeb* and *Atg3* in order to repress them through chromatin reorganization, including H3K4me2 demethylation[55]. In the neuroblastoma cell line Tet-21/N, LSD1 directly binds and represses the *Sestrin2* gene to facilitate H3K4me2 demethylation and other repressive chromatin changes[56]. This leads to SESN2 repression, promotion of mTORC1 activity, suppression of autophagy, and thus normal cell homeostasis[56]. LSD1 also repressed autophagy by promoting mTOR signaling in the ovarian cancer cell line HO8910[57]. In gynecologic cancer cell lines, LSD1 directly interacts with and destabilizes p62, a selective autophagy substrate, to suppress autophagy by preventing p62-LC3 interaction[58]. In castration-resistant prostate cancer cell lines, LSD1 suppressed autophagy through an unidentified mechanism, possibly contributing to

cell survival[59]. Five of the above studies[56-60] and one by Wang et al.[60] highlight that pharmacological LSD1 inhibition in multiple cancer cell lines induce autophagy through overexpression of corresponding genes, H3K4me accumulation, cell cycle arrest, and apoptosis, which has implications for treatment of cancer and diseases that have an autophagy component.

Cell Cycle

During cell proliferation, the expression of hundreds of genes is coordinately regulated with cell cycle phases so that cellular functions during specific phases can be carried out[61]. LSD1 has been shown to facilitate the coordinated cell cycle regulation of numerous genes, the first set of genes being those encoded by the mulitcistronic transcript at the Epstein-Barr virus latency promoter Cp[62]. Using either Burkitt's lymphoma cell lines (MutuI, Raji) or an EBV-transformed lymphoblast cell line, the Cp-encoded transcript was shown to be upregulated 2.5-fold in S phase while an RB1-LSD1 complex directly bound Cp following S-phase to demethylate H3K4me2 and decrease its expression. In mouse embryonic stem cells (ESCs), LSD1 was directly recruited to chromatin during G_1/S/G_2 phases, but was displaced into the cytoplasm during M phase[63]. Corresponding ChIP-seq datasets showed that LSD1 directly bound ESC gene promoters methylated by H3K4me2, such as *Oct4* and *Sox2*, corroborating previous work in ESCs[64]. A follow-up study found that this chromosomal displacement during M phase was due to PLK1-mediated phosphorylation of LSD1 at Ser126[65]. Functionally, LSD1 promoted chromosomal segregation during mitosis in HeLa and U2OS cells, which was partially due to transcriptional activation via H3K9me1 demethylation of the key mitosis mediators

Bubr1 and *Mad2*[66]. Upon LSD1 knockdown, cells in the G_1 phase decreased while cells in the G_2/M phases accumulated, suggesting that LSD1 promoted the G_1/S phase transition. Via a separate mechanism, LSD1 was found to promote S phase entry by forming a complex with the S phase transcription factor E2F1 and demethylating H3K9me2 at target cell cycle genes in a prostate cancer cell line[67]. LSD1 was also found to support E2F transcription factor activity by directly demethylating MYPT1, a regulator of phosphorylated RB1 levels, which decreased MYPT1 stability and thus increased phosphorylated RB1 levels in HEK293 cells[21]. These cell cycle-based analyses provide mechanistic insights into how LSD1 drives cell cycle progression in normal and diseased tissue.

Embryogenesis

The first LSD1 deletion mouse was published in 2007 and was achieved by flanking exon 6 of LSD1 with LoxP sites, recombination of which resulted in complete lack of protein[68]. Whole mouse deletion of LSD1 via this system resulted in developmental failure prior to embryonic day (E)7.5[68], providing the first piece of evidence that LSD1 is required for embryonic development. Mouse embryogenesis begins after an egg cell that is successfully fertilized by a sperm cell forms a zygote. Zygotic proliferation forms a 16-32 cell morula by E3, then a blastocyst containing an inner cell mass (ICM) within a trophoblast by E4. By E5-6, the blastocyst will implant into the uterine epithelium and the ICM will undergo gastrulation to form the ectoderm, mesoderm, and endoderm[69]. Examination of LSD1 expression during this process showed that it is first expressed at the E3 morula stage and is later expressed in the ICM and trophectodermal cells of

blastocysts[19]. LSD1-deficient embryos generate an egg cylinder, but fail to elongate and gastrulate, resulting in embryo resorption[19]. Prior to faulty gastrulation, LSD1 deficiency resulted in significantly increased quantities of embryonic basement membrane, suggesting an aberrant expansion of the parietal endoderm and implicating LSD1 as a mediator of cell lineage allocation[70]. Conditional deletion of LSD1 highlighted a critical role during the development of the epiblast and trophoblast compartments[71]. Cultured mouse embryonic stem cells (ESCs) lacking LSD1 proliferate normally, but exhibit a severe defect in differentiation due to increased apoptosis and faulty cell cycle progression[19,72].

LSD1 regulates ESC gene expression through indirect and direct mechanisms. LSD1 facilitates Dnmt1 stability by demethylating it, thus promoting global DNA methylation and gene repression[19]. LSD1 also directly represses gene expression by decommissioning enhancers via H3K4me1 demethylation through interaction with a NuRD protein complex containing histone deacetylases and transcription factors such as OCT4 and NANOG[64]. LSD1 demethylates H3K4me2 at bivalent promoters bound by NuRD such as the promoters of *Foxa2* and *Eomes*, both critical regulators of the endodermal and mesodermal lineages[73]. Further direct regulation of target genes by LSD1 may be facilitated by its interaction with CoREST2, which is the predominant CoREST transcription factor expressed in ESCs[74]. LSD1 deletion in ESCs results in the aberrant upregulation and downregulation of hundreds of genes involved in processes such as anterior/posterior patterning, limb development, and general maintenance of ESC identity[64,72]. Importantly, LSD1 is a direct repressor of key ESC genes such as *Tbxt* (Brachyury), a regulator of mesoderm formation, and *Sox2*, a regulator of ESC pluripotency[64,72]. Additionally, LSD1 is associated with demethylation of H3K4me at long

terminal repeat (LTR) regions mapping to MERVL retrovirus sequences and zygotic genome activation genes including *Zscan4*, *Tcstv1*, and *Tcstv3*, likely contributing to their repression[70]. In mouse trophoblast stem cells cultured at E3.5, LSD1 prevented premature differentiation and migration and promoted proliferation. Regulation of migration was due to repression of the transcription factor OVOL2 via demethylation of promoter H3K4me1/2. These studies collectively identify LSD1 as being critical for multiple aspects of embryonic development and differentiation through the epigenetic repression of the ESC fate gene program.

Hematopoiesis and Blood Cell Function

Most blood cells are derived from multipotent hematopoietic stem cells (HSCs) that reside in the bone marrow. HSCs will differentiate into common myeloid progenitors or common lymphoid progenitors, which will give rise to the myeloid cell lineage and lymphoid cell lineage, respectively. LSD1 function is implied throughout this process at multiple stages, including HSC proliferation, early HSC differentiation, and later development and differentiation of both myeloid and lymphoid lineages.

The first study linking LSD1 to hematopoiesis was in 2007 when Saleque *et al*. showed that an LSD1-CoREST complex interacted with GFI1B, a key transcription factor that mediates HSC differentiation, to repress GFI1B-target genes by demethylating H3K4me2 at their promoters[75]. Target genes of this complex include *Gfi1b* itself and the hematopoiesis transcription factor *Myb*. This LSD1-based function is essential for differentiation of hematopoietic cell lines into erythroid, megakaryocytic, and granulocytic cells, as well as primary erythroid progenitors. LSD-CoREST was also shown to interact

with TAL1 to mediate gene repression by the same mechanism and promote *in vitro* erythroid differentiation[76]. Additional work on the K562 erythroleukemia cell line showed that LSD1 recruited the BHC complex to GFI1B to promote erythroid differentiation[77].

In 2012, Diehl *et al*. utilized an inducible LSD1 knockdown mouse to probe the *in vivo* effect of LSD1 depletion on HSC homeostasis and differentiation[78]. LSD1 knockdown resulted in expanded populations of HSCs and lineage progenitors due in part to a prolonged G_0-G_1 transition. HSC differentiation into granulocytes and erythrocytes was decreased, while differentiation into monocytes and megakaryocytes was increased, with LSD1-depleted megakaryocytes being highly dysmorphic and unable to maintain normal platelet levels. Possible gene regulatory defects explaining these phenotypes include overexpression of key hematopoiesis transcription factors *Gfi1b*, *Hoxa9*, and *Meis1*, as well as aberrant repression of *Ly76* (Ter119), *Eng* (CD105), *Bcl2l1* (Bcl-χ_L), *Cebpa*, and *Elane*. In 2013, Kerenyi et al. utilized Vav1-Cre and Mx1-Cre recombinase systems to conditionally delete LSD1 in HSCs, corroborating the above results and also showing that LSD1 is important for HSC self-renewal and represses HSC genes during differentiation through H3K4me1/2 demethylation of enhancers and promoters[79].

To further characterize the *in vivo* role of LSD1 in the development and differentiation of blood cell lineages, conditional LSD1 deletion mice were generated with cell type-specific Cre recombinases. Both Cγ1-Cre and CD19-Cre was used to study the effect of LSD1 deletion in germinal center B cell differentiation, showing that it is necessary for their formation in response to T-dependent antigen[80]. Within germinal center B cells, LSD1 represses key plasma cell genes such as *Prdm1* (encodes Blimp-1) and *Irf4* through H3K4me1 demethylation at enhancers mediated by interaction with BCL6. In

developing T cells, LSD1 directly binds GFI1 to demethylate K370 and k372 of p53 to attenuate K177 acetylation and reduce pro-apoptotic p53 transcriptional activity[81]. Further work is needed to fully characterize the role of LSD1 in the remaining blood cell lineages.

Inflammation

LSD1 is involved in regulating inflammation, which is defined as a cellular response to certain homeostatic perturbations, including pathogens or damaged cells, resulting in processes aimed to ameliorate the perturbation, such as cytokine secretion[82]. LSD1 was first implicated in regulating inflammation in 2009 when Saijo *et al*. showed that LSD1 is required for Nurr/CoREST-mediated repression of inflammatory genes including *Nos2*, *Csf1*, and *Ncf1* in microglia and astrocytes, suggesting that LSD1 functions with Nurr1/CoREST to protect neurons from inflammation-induced death[83]. LSD1 may play a role in promoting an inflammatory environment in white adipose tissue, as LSD1-based repression of the inflammation-associated gene *Il6* was found to occur in differentiating 3T3-L1 preadipocytes, and white adipose tissue of obese mice exhibited decreased LSD1 expression and increased inflammatory gene expression[84]. Using a poly I:C inducible LSD1 deletion mouse strain via Mx-Cre, Wang *et al*. showed that LSD1 protects against endotoxic shock, as deletion following poly I:C injection causes endotoxic shock-like phenotype due to the expansion of a hyperproliferative and hyperinflammatory immature myeloid blast cell lineage in the bone marrow[85]. The LSD1-deficient HSCs that give rise to this lineage dysregulate hundreds of genes, including upregulation of *Gfi1b*[85], corroborating its known role in regulating gene expression during hematopoiesis[78]. During LPS-induced inflammation and acute lung injury in mice, the inflammatory response is

directly activated by a PKCα-LSD1-NFκB signaling axis[24]. LPS-stimulated bone marrow-derived macrophages were used to identify the specific mechanism: inflammatory stimuli facilitate PKCα translocation to the nucleus where it phosphorylates LSD1, phosphorylated LSD1 demethylates the NF-κB transcription factor p65 to enhance its stability, and demethylated p65 robustly activates the expression inflammatory genes such as *Il6* and *Il1b*[24]. LSD1 was also shown to promote renal inflammation associated with HBV infection[86]. Cell culture and mouse model experiments imply that this occurs through LSD1-mediated activation of *Tlr4* expression via H3K9me1/2 demethylation and suggest that HBV-associated renal inflammation can be attenuated by treatment with an LSD1 inhibitor such as TCP[86]. Overall, LSD1 regulates different inflammation-related pathways in different cell types, highlighting its importance in regulating adult homeostasis.

Inner Ear Development

LSD1 is a critical regulator of the differentiation and maintenance of inner ear progenitors. Early inner ear development is characterized by several important stages of embryogenesis: 1) otic-epibranchial progenitors (OEPs) form the otic placode; 2) the otic placode invaginates and separates to form an epithelial sac known as the otic vesicle or otocyst; 3) neuroblasts originating from the otocyst ventral region form the statoacoustic ganglion (SAG), which later innervates inner ear sensory structures; 4) the otocyst continues to develop and form key auditory and vestibular sensory structures including the organ of Corti, sacculus, utriculus, three cristae, and the endolymphatic duct and cochlear duct, with the latter housing the organ of Corti[87]. Recently, LSD1 was found to be expressed throughout the mouse otocyst at E9.5[88]. Using the VOT-N33 cell line derived

from auditory neuroblasts from the otocyst ventral region at E10.5, it was found that LSD1 forms a corepressor complex with PAX2 and NuRD at PAX2 genomic binding sites[88]. PAX2-LSD1-NuRD functioned to maintain otic progenitor identity, likely through the repression of key neuronal genes such as *NeuroD1* and *Ngn1* via H3K4me1/2 demethylation[88]. Another study revealed that LSD1 is also critical for otic placode formation from otic-epibranchial progenitors (OEPs) in developing chickens[89]. Through interaction with the transcription factor cMyb, LSD1 directly activated key otic genes such as *Sox8*, *Pax2*, *Etv4*, and *Zbtb16* in OEPs through H3K9me2 demethylation, thus maintaining OEP identity and preventing otic placode formation.

Myogenesis and Muscle Cell Function

Skeletal muscle progenitor cells, also known as myoblasts, differentiate into myocytes which fuse to form myofibers that make up muscle tissue[90]. Myoblast differentiation is a highly regulated process involving the key transcription factors MyoD and Mef2A-D[91]. In 2010, LSD1 was found to promote the differentiation of *in vitro*-derived myoblasts, directly interact with MyoD, Mef2C, and Mef2D, and target promoter H3K9me2 to activate myogenic genes such as *Myog* and *Ckm*[92]. Further *in vitro* work showed that LSD1 presence at the core enhancer region of the *Myod* gene locus led to demethylation of H3K4me1, H3K9me2, and H3K9me3, which in turn activated the expression of both *Myod1* and an eRNA transcript[93]. Crossing a floxed LSD1 mouse strain with the Pax3-Cre mouse strain ablated expression of *Lsd1*, *Myod1*, and its eRNA in forelimb muscle cell progenitors, which ultimately delayed myogenesis[93]. In addition to targeting histone modifications, LSD1 directly demethylates MEF2D to activate its

transcription factor activity by enabling recruitment to chromatin[22]. LSD1 also promotes myoblast differentiation by repressing non-myocyte master regulatory transcription factors, such as the osteoblast transcription factor RUNX2 via enhancer H3K4me1 demethylation[94].

LSD1 regulates muscle regeneration and metabolism. Upon tissue damage, muscle regeneration is facilitated by LSD1-dependent satellite cell differentiation into myocytes[95]. Mechanistically, LSD1 promotes myocyte cell fate by repressing the pro-adipogenic transcription factor GLIS1 and activating myogenic gene expression. LSD1 regulates metabolic programming in myocytes by binding and repressing oxidative metabolism genes, likely through direct and indirect modulation of H3K4me levels[96]. *In vivo* analyses indicated that muscles undergo glucocorticoid signaling-mediated degradation of LSD1 to induce expression of oxidative metabolism genes, revealing a molecular mechanism behind glucocorticoid-based regulation of muscle function. It will be interesting to see if LSD1 promotes additional muscle cell functions given its known interactions with integral myoblast differentiation transcription factors.

Neurogenesis and Neuron/Brain Function

The mammalian central nervous system contains several differentiated cell types, including neurons, astrocytes, and oligodendrocytes. LSD1 has been found to play a prominent role in the development of neurons, which function to relay information to other cells in the form of neurotransmitters or an electrochemical action potential. LSD1 plays an essential role in promoting neural stem cell proliferation both *in vitro* and *in vivo*[97,98]. In Y79 retinoblastoma cells and primary mouse neural stem cells, LSD1 is recruited by the

nuclear receptor TLX to repress target genes, such as *Cdkn1a* and *Pten*, through promoter H3K4me2 demethylation. CoREST, the CoREST-LSD1 interacting domain, and LSD1 itself were all necessary to promote the development of pyramidal cortical neurons at embryonic day 14.5 of mice[99]. LSD1 also facilitates the *in vitro* differentiation of human fetal neuronal stem cells by directly associating to and demethylating H3K4me2 at the promoter of *Heyl*, which is a Notch-target transcription factor[100]. Conversely, LSD1 was found to be depleted during mouse ESC commitment to neural progenitors, and its depletion promotes differentiation of the human neuroblastoma-derived SH-SY5Y neuronal cell line and mouse cortical neurons at embryonic days 13.5 to 15.5[101]. This effect was found to be dependent on interaction with JADE2, an E3 ubiquitin ligase, which ubiquitinates and thus targets LSD1 for degradation to prevent repression of genes that promote neuron differentiation such as *Pax3* and *Neurog1* through H3K4me1/2 demethylation at their promoters.

Further insight into the mechanistic role that LSD1 plays during neurogenesis was achieved by examining LSD1 isoforms. The exclusion, single inclusion, or double inclusion of the two LSD1 alternative splicing exons E2a and E8a leads to the expression of four different LSD1 proteins[39]. Native LSD1 and LSD1 spliced with exon E2a (LSD1+2a), a 60 bp exon encoding 20 amino acids that localize between the N-terminal disordered region and SWIRM domain, are expressed in all human tissue[39]. LSD1 spliced with exon E8a (LSD1+8a), a 12 bp exon encoding 4 amino acids that localize within the amine oxidase domain, or with both E2a and E8a (LSD1+2a+8a), are expressed specifically in brain tissue (LSD1+8a and LSD1+2a+8a) and testis (LSD1+2a+8a), with both isoforms being significantly upregulated in early developing rat brains[39]. Neurite

morphogenesis, or the formation of neuronal projections, is delayed or induced upon knockdown or overexpression of the neuron-specific LSD1 isoform LSD1+8a, respectively, during *in vitro* differentiation of rat cortical neurons[39]. This LSD1+8a-specific effect requires Thr369b of the 8a exon to be dephosphorylated, which causes detachment of corepressor proteins CoREST and HDAC1/2 and thus makes LSD1+8a functionally unable to repress the transcription of genes that promote neuron differentiation and function, including *Cdk5r1*, *Cdk16*, *Dlg4*, *Egr1*, *Fos*, and *Grin1*[102]. Despite evidence showing that LSD1+8a mediates gene repression through H3K4me1/2 demethylation[102], a later study showed that in the context the SH-SY5Y neuroblastoma cell line, LSD1+8a cannot intrinsically demethylate H3K4me2 but instead demethylates H3K9me2 through cooperation with the protein SVIL[38]. This demethylation occurs through LSD1+8a and SVIL association to the promoter regions of target genes, such as *BUB1B*, *CHRM3*, *CTDB1*, *DOCK9*, *ENOX2*, *TMTC3*, *UBR2*, and *ZCCHC8*, which facilitates gene activation and promotes SH-SY5Y differentiation.

In terms of brain function, alterations in levels of the LSD1-target histone modification H3K9me2 in brain tissue was correlated to memory formation in response to fear conditioning in mice[103]. Demethylation of H3K9me2 was later linked to LSD1 activity, as LSD1 inhibition with *trans*-2-phenylcyclopropylamine (TCP) enhanced fear conditioning and resulted in increased H3K9me2 at the *G9a* promoter in the lateral amygdala via NMDAR-ERK-dependent signaling[104]. Treatment of mice with RN-1, a more selective LSD1 inhibitor, has been shown to decrease long-term memory formation while leaving short-term memory formation uncompromised[105]. It is unclear whether or

not LSD1 inhibition with RN-1 is directly affecting neuron function or the function of other cell types.

Genetic mouse models have been used study the role of LSD1 in brain function. The *Lsd1*$^{SA/SA}$ knock-in mouse, which encodes an LSD1 protein unable to be phosphorylated at serine 112 by PKCα[106], has been used to study LSD1 brain function due to the known role of PKCα regulating the murine circadian rhythm[107]. *Lsd1*$^{SA/SA}$ knock-in mice exhibit impaired behavioral adaptation to photic stimuli, as well as defective CLOCK:BMAL1-mediated transcriptional activation of circadian rhythm genes, which was independent of LSD1 enzymatic activity[106]. In a separate study, *Lsd1*$^{SA/SA}$ knock-in mice were shown to have impaired short-term memory, hippocampus-dependent spatial memory, and social recognition memory[108]. Mechanistically, Lsd1$^{SA/SA}$ knock-in mice displayed defective short-term synaptic plasticity in the hippocampal CA1 region and hippocampus cells displayed significantly increased expression of the presynaptic function-related genes *Crhr1*, *Drd2*, *Hrh1*, *Hrh3*, *Rab39*, *Slc18a2*, and *Syngr1*. To examine the role of LSD1+8a in brain function, a neuron-specific conditional LSD1+8a deletion mouse was generated[16]. In cortical neurons, LSD1+8a-mediated demethylation of gene body H4K20me1 promoted transcriptional elongation and expression of neuron function genes such as *Npas4*, *Arc*, and *Egr1*. Functionally, LSD1+8a promoted spatial learning and long-term memory formation. Moreover, deletion of LSD1+8a was also shown to protect against pharmacologically-induced seizures[109] and cause a low-anxiety behavioral phenotype[110], the latter being facilitated by interaction with the transcription factor SRF and aberrant repression of neuron function genes such as *Egr1* and *Fos* in hippocampal cells, implicating an important role for LSD1+8a in neuron activation. It will

be important to examine the dynamic expression and gene regulatory mechanisms of LSD1 and its isoforms in the context of human brain tissue to fully discern its *in vivo* biological function during human neuron development and function.

Spermatogenesis

LSD1 plays a key role in facilitating spermatogenesis, which is the process by which spermatogonia, a self-renewing stem cell lineage in the testes, differentiate to form haploid spermatozoa that are able to fertilize oocytes[111]. During mouse spermatogenesis, H3K4me1/2 is highly dynamic and correlate with protein expression of LSD1[112]. Within purified mouse germ cells, LSD1 directly interacts with HDAC1 and MBD2a/b, suggesting the formation and function of an LSD1-based corepressor complex[112]. In the male germ cell line GC-1, inhibition of LSD1 activity with TCP and HDAC activity with trichostatin A (TSA) resulted in increased expression of *Pou5f1* (OCT4) and *Gfra1*, key mediators of spermatogenesis, as well as increased H3K4me2 and H3K9ac at these genes, implying LSD1-HDAC complex activity[113]. Experiments with germ cell-conditional LSD1 deletion mice via *Ddx4*-Cre have shown that LSD1 is required for spermatogonia maintenance and differentiation starting at 6 days post-partum, resulting in germ cell apoptosis and complete loss of germ cells by 21 days post-partum[114,115]. Inducible deletion of LSD1 in adult mice using the Cagg-Cre system showed a similar ablation pattern, supporting that LSD1 is necessary for the maintenance of spermatogonia[114]. At 6 days post-partum, LSD1-deficient spermatogonia exhibited increased global H3K4me2 levels and a spermatogonia/progenitor enriched population exhibited significant alterations in genes critical for spermatogonia function, including upregulation of *Bcl6b*, *Cxcr4*, *Sohlh2*, *Cers3*,

Spink2, *Insl6*, and *Syce3* and downregulation of *Plzf*, *Sall4*, *Pou3f1*, *Nanos2*/*3*, *Ddit4*, and *Lin28a*[115]. In whole testis, LSD1 binds the *Oct4* locus and likely functions to demethylate H3K4me2[114]. Overall, LSD1 plays an epigenetically and transcriptionally repressive role to promote spermatogenesis function, possibly through the activity of multiple key target genes.

Other Pathways

In 2007, Wang et al. showed that by conditionally deleting LSD1 using the pituitary-specific Pitx1-Cre mouse, pituitary glands form normally but hormone-secreting cell types that arise from pituitary progenitors, such as somatotropes, thyrotropes, and lactotropes, fail to properly develop[68]. LSD1 regulated multiple aspects of pituitary gene regulation at embryonic day E17.5 in $Pit1^{+}$ progenitors, including 1) activation of Pit1-target genes *Prl*, *Tshb*, *Gh1* (growth hormone), and *Pit1* itself via Pit1 interaction; 2) repression of Notch-target gene *Hey1* via interaction with Notch-interacting transcription factor RBP-J; and 3) repression of cell cycle genes *Ccne1* and *Id2* via interaction with a CoREST-CtBP-containing corepressor complex and demethylation of promoter H3K4me2. LSD1 later functions in complex with a ZEB1-CoREST-CtBP complex to repress growth hormone gene expression postpartum. In the MEF cell line, LSD1 was shown to facilitate the repression of the Notch-target gene as a member of the SIRT1-LSD1-CtBP complex, validating a relationship with Notch and suggesting that LSD1 may function in other Notch-mediated developmental pathways[116]. In Drosophila, the LSD1 ortholog dLSD1 was found to facilitate normal development of posterior cross vein

patterning on the wing and anterior scutellar bristles on the notum, demonstrating the conserved ability of LSD1 to regulate Notch-mediated developmental pathways[116].

A multitude of other studies identified additional functions of LSD1. In mouse olfactory sensory neurons (OSNs), LSD1 is required for the initial expression of olfactory receptors (ORs) and subsequent targeting of the OSN axons during embryonic development[117]. In the human duodenum adenocarcinoma cell line HuTu 80, LSD1 interacts with CtBP and RREB1 to activate the gene encoding the hormone secretin via H3K9me2 demethylation, implying a possible biochemical role for LSD1 in regulating GI tract function[118]. LSD1 was implicated in regulating bile acid metabolism through experiments performed on liver hepatocytes[119]. Upon activation of FXR, the primary bile acid receptor, LSD1 increases in expression and is recruited by SHP to BA synthetic genes *Cyp7a1* and *Cyp8b1* and the BA uptake transporter gene *Ntep* to facilitate gene repression, thus mediating the natural bile acid negative feedback loop[119]. To promote the differentiation of primary human epidermal keratinocytes, a ZNF750 complex containing LSD1, CoREST, and CtBP1/2 are recruited to progenitor genes to induce repression via H3K4me1 demethylation[120]. Based on the studies above, it is becoming increasingly clear that LSD1 has key functions throughout the development and differentiation of many different cell types. It is also clear that LSD1 is critical for the function of many fully differentiated cell types in order to maintain proper homeostatic conditions.

Disease and its Treatment

Cancer

The study of LSD1 in cancer has stemmed from the observation that LSD1 is highly expressed in multiple cancer cell types (**Table 1**) and, for certain cancers, is associated to poor clinical outcomes. The importance of LSD1 in maintaining cancer growth and survival is highlighted in the numerous preclinical studies and clinical trials showing that LSD1 inhibition can ablate certain cancers (see "Pharmacological targeting" section). Studying the role of LSD1 in cancer through cell lines, primary samples, and mouse models has led to novel insights into the biological functions of LSD1, how epigenetic modifying enzymes can promote cancer growth, and how cancer can be therapeutically targeted. These findings are not discussed here, as this topic has been heavily reviewed elsewhere[3,4,121-140]. In summary, LSD1 promotes cancer growth through different mechanisms that alter gene expression in favor of cancer growth. For example, in castration-resistant prostate cancer, LSD1 was found to promote the expression of genes that drive lethal prostate cancer malignancy[141] through interaction with ZNF217 in a demethylase-independent manner[142]. A different example is seen in acute myeloid leukemia (AML), where LSD1 has been shown to form a complex with GFI1 and RCOR1 to preserve the AML transcriptional identity by repressing transcription factors associated with blast cell differentiation[143]. These examples are a few out of many that highlight the complex role that LSD1 has in regulating cancer formation and progression. Further research will be important for identifying additional novel LSD1-based mechanisms that can be pharmacologically targeted for cancer treatment.

Table 1: Human cancer types that exhibit high expression of *KDM1A* compared to normal tissue

Type of Cancer	**Citation**

Prostate cancer	Metzger et al. 2005[15], Kahl et al. 2006[144], Wissmann et al. 2007[145], Kashyap et al. 2013[146], Etani et al. 2019[59]
Brain cancer	Schulte et al. 2009[147], Pajtler et al. 2013[148], Ambrosio et al. 2017[56]
Lung cancer	www.oncomine.org[149], Hayami et al. 2011[150], Lv et al. 2012[151], Mohammad et al. 2015[152]
Breast cancer	Lim et al. 2010[153], Serce et al. 2012[154], Wu et al. 2013[155], Nagasawa et al. 2015[156], Cao et al. 2017[157]
Bladder cancer	www.oncomine.org[149], Hayami et al. 2011[150], Kauffman et al. 2011[158]
Gastric cancer	Fang et al. 2017[159], Zhang et al. 2019[160]
Ovarian cancer	Konovalov et al. 2013[161], Chen et al. 2015[162], Chao et al. 2017[58], Tsai et al. 2018[163]
Colorectal cancer	www.oncomine.org[149], Hayami et al. 2011[150], Ding et al. 2013[164], Huang et al. 2013[165], Jie et al. 2013[166]
Hematopoietic malignancies	www.oncomine.org[149], gepia.cancer-pku.cn[167], Radich et al. 2006[168], Goardon et al. 2011[169], Niebel et al. 2014[170]
Mesothelioma	www.oncomine.org[149]
Pancreatic cancer	gepia.cancer-pku.cn[167], Qin et al. 2014[171]
Endometrial cancer	Chao et al. 2017[58], Tsai et al. 2018[163]
Sarcoma	Bennani-Baiti et al. 2012[172], Pishas et al. 2018[173]
Thymoma	gepia.cancer-pku.cn[167]
Oral cancer	Narayanan et al. 2015[174], Yuan et al. 2015[175], Alsaqer et al. 2017[176]
Esophageal cancer	Yu et al. 2013[177]
Liver cancer	Zhao et al. 2012[178], Zhao et al. 2013[179], Sakamoto et al. 2015[180]

Genetic Disease Resulting from *KDM1A* Mutation

Due to the importance of LSD1 in multiple different pathways, mutations in the *KDM1A* gene, which encodes LSD1, can be detrimental to human health. By 2015, three individuals (males that were 3-years-old, 4-years-old, and 8-years-old at the times of publication) were reported to have likely deleterious heterozygous mutations in *KDM1A*[181-183]. Each mutation was identified through exome sequencing as a unique *de novo* missense point mutation within the LSD1 amine oxidase domain, and all were not present in any of the 71,000 control exomes analyzed[181]. Each proband exhibited a multitude of atypical phenotypic characteristics, including intellectual disability, developmental delay, and

physical defects such as craniofacial abnormalities[181]. A follow-up study in 2016 examined the functional consequences of all three reported *KDM1A* deleterious heterozygous mutations[184]. Each mutation affects the enzymatic active site and recombinantly expressed, purified mutant LSD1 proteins are partially impaired in their ability to catalyze demethylation of H3K4me1[184]. Furthermore, all mutant LSD1 proteins displayed significantly reduced cellular half-lives while two displayed reduced active site binding to the histone-mimicking transcription factor SNAIL1[184], suggesting additional mechanisms of impaired function. These studies not only provide insight into the multiple roles of LSD1 in human development, but also highlight the significant differences between mouse and human genetic studies, as mice heterozygous for *Kdm1a* develop and reproduce normally[68]. Studies examining the role of germline LSD1 mutations in developmentally normal individuals will be important as well. One example of autosomal dominant truncating LSD1 mutations has already been found to confer susceptibility to early-onset multiple myeloma[185].

Neurodegenerative Disease

LSD1 may play a protective role against neurodegenerative diseases, which are diseases that cause progressive loss of cognitive and/or motor function[186]. Adult mice that underwent full body deletion of LSD1 via the tamoxifen-inducible CAGG-*Cre*-ER^{TM} system exhibited significant neurodegeneration in the hippocampus and cerebral cortex in association with motor defects, learning and memory defects, and eventual death[187]. LSD1-deficient hippocampal cells displayed derepression of stem cell genes, such as *Klf4*, *Myc*, *Foxo1*, and *Oct4*, and induction of genes involved in human neurodegenerative

pathways, such as *Tyropb*[187]. In the brains of humans with neurodegenerative disease, LSD1 was both normally localized in neuronal nuclei and mislocalized in cytoplasmic aggregates and neurites, and hippocampal neurons displayed increased expression of stem cell gene expression, similar to the mouse model[187]. Contrary to the above, LSD1 has been shown to repress autophagy[54-60], which has a protective effect against neurodegeneration because of its role in degrading protein aggregates[188]. Indeed, LSD1 deletion in C. *elegans* was shown to suppress neurotoxicity associated with misfolded proteins[54]. Oxidative stress-induced death of primary rat cortical neurons was reduced with treatment of the LSD1 inhibitor bizine[189], suggesting that oxidative stress-associated neurodegeneration[190] may be countered with LSD1 inhibition. Furthermore, both NMDA-induced excitotoxicity and oxidative stress-induced death of rat retinal ganglion cells was reduced by treatment with the LSD1 inhibitor TCP[191]. Further work taking into account both the positive and negative effects of LSD1 inhibition and ablation in human neurons will be needed before developing an LSD1-based treatment targeting neurodegeneration.

Sickle Cell Disease

LSD1-based treatments have been explored as a treatment for sickle cell disease (SCD), which is a group of genetic red blood cell disorders characterized by sickle-shaped red blood cells due to faulty hemoglobin (Hb, $\alpha_2\beta_2$) protein, resulting in pain, swelling, anemia, organ damage, and other symptoms[192]. Fetal hemoglobin (HbF, $\alpha_2\gamma_2$), the main form of hemoglobin in human infants less than six months of age, can be pharmacologically reactivated in order to replace faulty Hb and decrease SCD severity[193]. LSD1 inhibition was tested as a method to increase HbF levels due to its known role in functioning within

corepressor complexes containing transcription factors NR2C1/2 and BCL11A to suppress HbF expression in adult erythroid cells[194,195]. In *ex vivo*-differentiated $CD34^+$ primary human progenitor cell cultures, LSD1 inhibition via TCP resulted in a significant increase in γ-globin expression and H3K4me2 accumulation at its promoter[196]. Treatment of adult mice with TCP induced γ-globin expression in bone marrow cells[196], while treatment of SCD mouse models with the selective LSD1 inhibitor RN-1 resulted in an increase in red blood cell HbF levels with a concomitant decrease in SCD pathology[197,198]. RN-1 treatment of adult anemic baboons, as well as adult and juvenile non-anemic baboons, resulted in increased γ-globin expression and HbF levels, likely due to a lack of H3K4me demethylation at the γ-globin gene[199,200]. Importantly, these experiments showed minor negative side effects when the proper RN-1 dosage was administered, suggesting positive clinical efficacy for treatment of SCD in humans[199,200] despite the known role of LSD1 in regulating multiple developmental and functional pathways. Further work recapitulated the above findings in *ex vivo*-differentiated CD34+ primary human progenitor cell cultures and SCD mouse models treated with the selective LSD1 inhibitors GSK-LSD1 and OG-L002, providing additional pharmacologic options to target LSD1 to treat SCD[201].

Viral Infection

Therapies targeting epigenetic modifiers to combat herpes simplex virus (HSV) infection have been explored due to the known role of chromatin modifications in regulating viral gene expression once the viral genome has integrated into the host. A role for LSD1 in regulating HSV gene expression was first shown in 2009 when Gu et al. demonstrated that LSD1 is partially degraded in a proteasome-dependent manner during

HeLa cell infection with HSV-1 and that LSD1/HDAC1/CoREST complexes localized with ICP8 in vicinity of ND10 bodies[202]. These findings suggested that LSD1 degradation is induced by HSV infection, possibly to prevent LSD1-based repression of its viral genome. Later that year, Liang et al. published findings that suggested a different mechanism of LSD1 function during HSV infection. They showed that the genomes of α-herpesviruses accumulate repressive H3K9 methylation following lytic infection[203]. In order to facilitate a transition from heterochromatin to euchromatin at herpesvirus genomes during reactivation from latency, the coactivator HCF-1 localizes to and recruits chromatin modifiers to HSV genomes, including LSD1, Set1, and MLL1[203,204]. LSD1 specifically demethylates H3K9me to activate viral immediate early (IE) gene expression, and treatment of cell line and mouse HSV infection models with the monoamine oxidase inhibitors TCP and pargyline, as well as the LSD1 inhibitor OG-L002, promotes the accumulation of repressive chromatin modifications, blocks viral IE gene expression, and reduces reactivation from latency[203,205]. Further *in vivo* work showed that treatment with TCP abrogated HSV infection in mouse oral and intranasal models, the rabbit eye model, and the guinea pig genital model through the same pathway[206].

Other viral infection cycles have been shown to utilize LSD1, suggesting that they can also be pharmacologically targeted by LSD1 inhibitors. Similar to HSV infection, cells treated with TCP or the selective LSD1 inhibitor OG-L002 following infection with human cytomegalovirus (hCMV) or adenovirus type 5 exhibited a reduction in IE gene expression[205,207]. Human immunodeficiency virus (HIV) reactivation from latency is promoted by the viral gene product Tat, and LSD1 has been shown to demethylate Tat at K51, thus promoting its ability to transactivate HIV gene expression[25]. The monoamine

oxidase inhibitor phenelzine was shown to suppress HIV reactivation from latency in a primary T cell model of HIV latency, solidifying an activating role for LSD1 during the HIV infection cycle[25]. LSD1 has also been shown to repress HIV-1 replication and transcription during infection of a human microglial cell line through interaction with the transcription factor CTIP2[208], suggesting an alternate LSD1 function during latency in HIV-infected microglial cells. Experiments examining hepatitis B virus (HBV) infection of the human hepatocarcinoma cell lines showed that transcriptional activation by the viral protein HBx is partially mediated by its recruitment of LSD1, which, similar to its role in HSV infection, demethylates H3K9me2 at viral gene promoters[209]. Via another distinct mechanism, LSD1 demethylates the host antiviral protein IFITM3 at K88 to promote its activity during infection with influenza A virus (IAV), which is demonstrated through the detrimental effects that TCP has during mouse IAV infection[26]. Overall, LSD1 has wide-ranging effects in regulating viral infection cycles and may represent a viable pharmacological target for treatment of viral infections.

Pharmacological targeting

Because epigenetic changes are reversible, diseases promoted by aberrant epigenetic changes can be treated with small molecules that alter epigenetic modifying enzyme function. LSD1 is not an exception, and numerous small molecules targeting LSD1 have been tested and used for treatment against a variety of human diseases. The first LSD1 inhibitors identified were the monoamine oxidase inhibitors (MAOIs) pargyline, deprenyl, and clorgyline, which were shown to inhibit LSD1-mediated H3K9me2 demethylation and downstream androgen receptor-mediated transcriptional

activation *in vitro*[15]. Further studies testing the ability of MAOIs to suppress LSD1-mediated H3K4me1/2 demethylation found that pargyline, deprenyl, clorgyline, and nialamide were unable to inhibit this function, while phenelzine and particularly tranylcypromine (TCP, also known as trans-2-phenylcyclopropylamine or 2-PCPA) exhibited strong inhibitory effects[35,210]. Mechanistically, TCP is an irreversible LSD1 inhibitor that covalently modifies LSD1-bound FAD to prevent catalytic activity[211,212]. More selective LSD1 inhibitors have been designed based off of MAOIs and include the phenelzine analogue bizine[189] and multiple TCP derivatives such as the anti-HSV inhibitor OG-L002[205] and the anti-cancer inhibitors GSK-2879552[152,213-215], GSK-LSD1[152,176,216], NCL-1[59,148,217-221], NCD-38[221-226], ORY-1001 (also known as RG6016)[4,216,227], RN-1[105,161,228], S2101[161,229,230], and T-3775440[231-233].

Due to inhibition of non-LSD1 proteins by MAOIs, screening methods employing techniques such as mass spectrometry[234,235], demethylation assays[236,237], heterogeneous immunoassays[238,239], FRET[240-242], scintillation proximity assays[243], microfluidic capillary electrophoresis[244], and virtual screening[245-249] have been developed and used to discover more selective LSD1 inhibitors. Peptide inhibitors that mimic the histone 3 tail have been shown to be efficacious in LSD1 inhibition[250-255]. However, peptide inhibitors have poor membrane permeability[256], thus their biological use is limited. The LSD1 inhibitory effects of polyamine analogues were explored due to high homology between LSD1 and the known polyamine analog inhibitory target spermine oxidase, as well as their structural similarity to the lysine tails of histones, which led to the discovery of several LSD1 inhibitors with anti-cancer effects[4,138,257]. Multiple selective, reversible inhibitors of LSD1 with potential therapeutic applications were generated. For example, the N'-(1-

phenylethylidine)-benzo hydrazide compound SP-2509[247] has been shown to impede the growth of prostate cancer cell lines[258], Ewing sarcoma cell lines[173], and neuroblastoma cell lines[259], as well as have preclinical efficacy in AML[260], endometrial cancer[261], Ewing sarcoma[262], lung adenocarcinoma[263], and castration-resistant prostate cancer[142]. Other compounds with LSD1 inhibitory effects include the natural products resveratrol, curcumin, quercetin, geranylgeranoic acid, and α-mangostin[264-266], resveratrol derivatives[267], a rhodium(III) complex[268], 5-hydroxypyrazoles[269], pyrimidine-thiourea hybrid molecules[270], and phenyl oxazoles[271], representing additional future drug leads.

Certain LSD1 inhibitors have undergone or are currently undergoing clinical trials for treatment of specific diseases. The MAOI TCP is FDA approved since it has been used to treat depression since the 1960s[272], and is also used to treat AML off-label[273]. Ongoing clinical trials are testing different treatment methods involving TCP against AML and other myeloid malignancies, such as using TCP in conjunction with the drug ATRA (ClinicalTrials.gov identifiers NCT02273102, NCT02717884, NCT03043105). Phenelzine, another MAOI known to target LSD1, is undergoing clinical trials to treat metastatic or advanced breast cancer in combination with the chemotherapeutic agent Abraxane (NCT03505528), as this combination was previously shown to be an effective treatment in a preclinical study[122]. The N-alkylated TCP derivative ORY-1001, first developed and patented by Oryzon Genomics[4,227], is highly selective for LSD1 over other MAOIs and has been shown to abrogate AML in a mouse xenograft model[274]. ORY-1001 is currently in European Union clinical trials for treatment of AML and small cell lung cancer (SCLC) (EudraCT 2018-000482-36, 2018-000469-35, 2013-002447-29) and, following an AML first-in-human phase 1 study, has been shown to be well tolerated at the

recommended dose and to promote blast cell differentiation in more than half of tested patients[275]. The TCP derivative GSK2879552 was found to inhibit the growth of SCLC cell lines and primary samples[152,215], as well as AML cell lines and primary samples[213,214]. Several clinical trials have been initiated to treat AML, myodysplastic syndrome (MDS), and SCLC with GSK2879552, however these trials have since been terminated due to unfavorable risk benefits (NCT02177812, NCT02929498, NCT02034123). The N'-(1-phenylethylidine)-benzo hydrazide compound SP-2577 (also known as HCI-2577 or Seclidemstat)[247], an analog of SP-2509, is currently undergoing clinical trials for treatment of Ewing sarcoma and other solid tumors (NCT03600649, NCT03895684). The irreversible LSD1 inhibitor IMG-7289 developed by Imago BioSciences has completed a phase 1 trial with ATRA to treat AML and MDS (NCT02842827) and has begun a phase1/2A trial to treat myelofibrosis (NCT03136185). At the American Association for Cancer Research (AACR) annual meeting in 2018, Incyte Corporation showed that their LSD1 inhibitor INCB059872 suppressed the growth of stem-like cells from mouse prostatic tumors and human prostate cancer cell lines[276] and induced myeloid differentiation in primary human AML samples[277]. INCB059872 is currently undergoing clinical trials for treatment of Ewing sarcoma, AML, MDS, SCLC, myelofibrosis, and neuroendocrine tumors (NCT03514407, NCT02712905), as well as the treatment of metastatic cancers in combination with the immune checkpoint drugs pembrolizumab and epacadostat (NCT02959437). Celgene Corporation developed the LSD1 inhibitor CC-90011, which is in clinical trials for treatment of Non-Hodgkin's lymphoma (NHL) and advanced solid tumors such as neuroendocrine tumors (NETs) and SCLC (NCT02875223,

NCT03850067). As of January 2018, the clinical trials for NETs and NHL have revealed preliminary evidence of antitumor activity, particularly with NETs[278].

The above studies and clinical trials not only display the extensive research that has led to the discovery and production of clinically relevant LSD1 inhibitors, but also lay the foundation for the future development of selective, non-toxic, bioavailable LSD1 inhibitors. Further research is needed to fully understand the long-term effects of prolonged LSD1 inhibition on human health, which may be a critical limiting factor in LSD1-targeted therapies. In addition to its enzymatic function, other important LSD1 functions, such as its ability to bind transcription factors, can be targeted for pharmacological inhibition, as demonstrated by the inhibitor SP-2509, which acts as an allosteric inhibitor of LSD1 and blocks its interaction with ZNF217 in prostate cancer cell lines[142]. Indeed, Hatzi et al. demonstrated through a CRISPR/Cas9 domain screen that, in addition to the amine oxidase domain, the tower domain of LSD1 is critical for promoting the growth of germinal center-derived lymphoma cells, emphasizing the importance of LSD1-mediated protein-protein interactions[80]. Screens that identify compounds capable of targeting the non-enzymatic functions of LSD1 will be important for developing novel inhibitors.

Chapter 1 Part B: B cell development and differentiation

B cell function

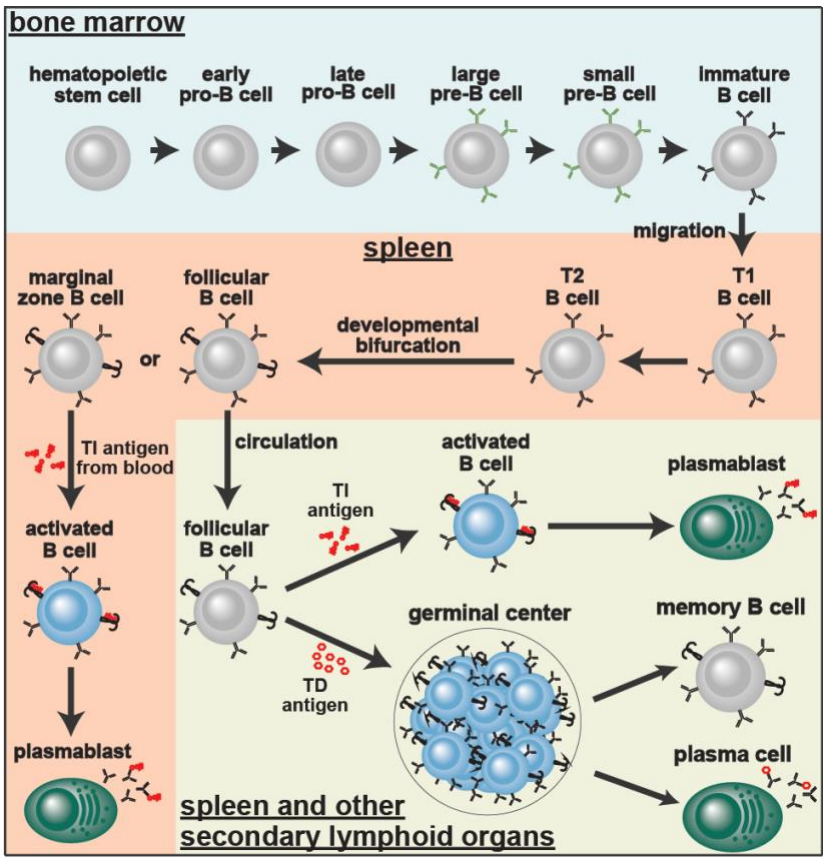
B cells are a type of white blood cell that circulate throughout the body and mediate an adaptive immune response against pathogens, such as bacteria, viruses, or parasites.

Following development from hematopoietic stem cells (HSCs), B cells interact with antigen and differentiate into antibody-secreting cells known as plasma cells. Depending on the antigen, the secreted antibody will either be polyreactive to multiple antigen or highly specific to a single antigen. Once antibody binds a pathogen, the pathogen's effectiveness is hindered through multiple mechanisms: 1) preventing interaction of the pathogen with its target cell, 2) facilitating opsonization and destruction by other white blood cells such as macrophages, 3) activating a complement cascade to further facilitate opsonization or promote pathogen destruction through forming membrane pores[279].

B cell development

Hematopoietic developmental pathways give rise to two distinct populations of B cells: B-2 cells and B-1 cells (**Fig. 1-3**). B-2 cells constitute the majority of B cells and arise primarily from HSCs from adult bone marrow (**Fig. 1-3 A**). Two types of B-2 cells exist: follicular B cells (FoB) and marginal zone B cells (MZB). FoB circulate throughout secondary lymphoid organs and respond to both T-dependent (TD) and T-independent (TI) antigen, generating long-lived plasma cells (PC), short-lived plasmablasts (PB), and memory B cells (MBC)[280]. MZB are restricted to the splenic marginal zone where they rapidly respond to bloodborne TI antigen to generate short-lived PB[281]. B-1 cells arise primarily from HSCs from the fetal liver and bone marrow[282] (**Fig. 1-3 B**). B-1 cells reside mainly in the serous cavities such as the peritoneal cavity, generate natural antibody via spontaneous secretion of polyclonal IgM that has protective and homeostatic functions, and rapidly respond to TI antigen by generating short-lived PB[282]. B-1 cells can be further

A


bone marrow
hematopoietic stem cell
early pro-B cell
late pro-B cell
large pre-B cell
small pre-B cell
immature B cell
migration
spleen
marginal zone B cell
or
follicular B cell
developmental bifurcation
T2 B cell
T1 B cell
TI antigen from blood
activated B cell
circulation
follicular B cell
TI antigen
activated B cell
plasmablast
germinal center
memory B cell
TD antigen
plasmablast
plasma cell
spleen and other secondary lymphoid organs

B

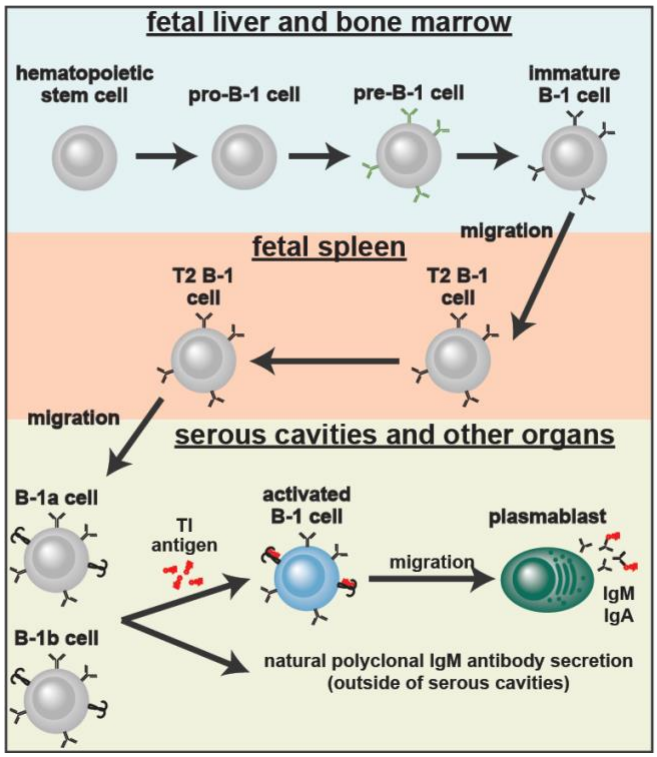

fetal liver and bone marrow
hematopoietic stem cell
pro-B-1 cell
pre-B-1 cell
immature B-1 cell
migration
fetal spleen
T2 B-1 cell
T2 B-1 cell
migration
serous cavities and other organs
B-1a cell
TI antigen
activated B-1 cell
plasmablast
migration
IgM
IgA
B-1b cell
natural polyclonal IgM antibody secretion
(outside of serous cavities)

Figure 1-3 – B cell developmental and differentiation pathways
(A) B-2 B cell development and differentiation. **(B)** B-1 B cell development and differentiation

subdivided into $CD5^+$ B-1a cells and $CD5^-$ B-1b cells, which differ in responses to specific pathogens[283].

Early B cell development is characterized by the formation of B cell receptor (BCR)-expressing immature B cells from hematopoietic stem cells[284]. Hematopoietic stem cells develop through early lymphoid progenitors, common lymphoid progenitors, then the B cell lineage-specific pro-B cell, where they undergo BCR heavy chain gene rearrangement. During the pro-B to pre-B cell transition, cells express the rearranged heavy chain with a non-polymorphic surrogate BCR light-chain protein to form the pre-BCR. Pre-BCR signaling in conjunction with cytokine signaling (ex: IL-7) promotes pre-B cell proliferation and survival. The BCR light-chain gene then undergoes rearrangement, and both heavy chain and light chain genes are expressed to form a functional BCR. At this point, the pre-B cell has progressed into an immature B cell.

To complete development, immature B cells migrate to the spleen where they undergo transitional B cell development[285]. Once they reach the spleen, they are considered transitional stage 1 (T1) B cells. T1 B cells participate in multiple signaling cascades, which drives a developmental bifurcation event. If T1 B cells receive weak tonic BCR signaling in addition to NOTCH2 signaling, they are primed to become marginal zone B cells. If T1 B cells receive strong tonic BCR signaling in the absence of NOTCH2 signaling, they are primed to become follicular B cells. Both cells must undergo non-canonical NF-κB signaling induced through the BAFF receptor. Canonical NF-κB signaling also seems to play a role, though to a much lesser extent.

B cell differentiation

B cells circulate throughout the body primarily in secondary lymphoid organs including lymph nodes and the spleen in search of antigen. B cells have two general responses to antigen, TI and TD, which are defined by the cellular help they receive from T cells, the other lymphocyte population in the body. In TI responses, B cells do not receive T cell help to complete activation and are instead fully activated by other signals such as BCR crosslinking and/or toll-like receptor (TLR) stimulation. TI antigens are typically non-protein and include the TLR4 agonist lipopolysaccharide and encapsulated bacteria with highly repetitive surface epitopes, which can induce BCR-crosslinking. Following activation by a TI antigen response, B cells will proliferate and differentiate into short-lived PB that primarily generate polyclonal IgM antibody.

In TD responses, B cells receive help from T cells in order to complete activation[286]. Specifically, B cells interact with TD antigen via the BCR, internalize the antigen and present it via surface MHC-II molecules, helper T cells bind the MHC-II-antigen complex via their T cell receptor (TCR) to become activated, and activated T cells express co-stimulatory molecules on their surface for B cells to interact with, such as CD40, to complete activation. Following T cell help, B cells will form germinal centers, which are aggregates of lymphocytes within secondary lymphoid organs that facilitate the generation of highly specific antibody-secreting plasma cells and memory B cells (**Fig. 1-4**). Within germinal centers, activated B cells circulate between the light zone and dark zone, during which they undergo multiple rounds of somatic hypermutation to introduce novel

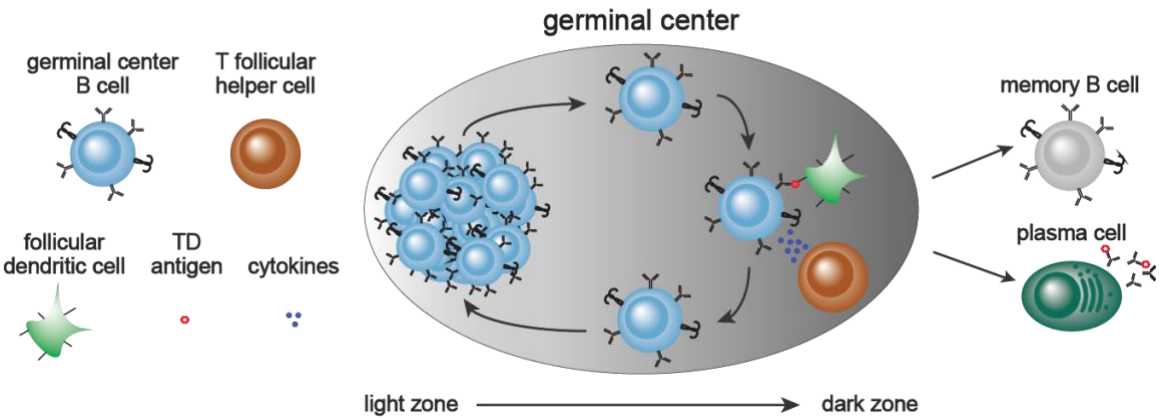


Figure 1-4 – Germinal center B cell differentiation
Germinal center B cell differentiation.

mutations within their BCR gene loci. Germinal center B cells also undergo isotype class switching from the default IgM heavy chain to one of three other heavy chains depending on cellular signaling events: IgA, IgG, IgE. With additional signals and interactions between B cells and T follicular helper cells and follicular dendritic cells, B cells with highly specific BCRs towards their target antigen are selected for survival and differentiation into long-lived PC and MBCs. PC migrate to the bone marrow, where they will reside for up to the hosts lifetime, all while secreting a high volume of antigen-specific antibody. MBCs will circulate throughout the secondary lymphoid organs, similar to naïve B cells, where they will much more rapidly differentiate into PC upon BCR stimulation with the same antigen.

Chapter 2: LSD1 regulates B cell proliferation and plasmablast differentiation

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Abstract

B cells must undergo division-dependent epigenetic remodeling of gene promoters and *cis*-regulatory elements to differentiate into antibody-secreting cells (ASCs). The master regulatory transcription factor BLIMP-1 reprograms the B cell epigenome during ASC formation by recruiting histone modifying proteins to genomic binding sites. LSD1 is a histone demethylase known to decommission active enhancers and cooperate with BLIMP-1. The specific contribution of LSD1 to ASC formation is poorly understood. To address this topic, *in vivo* LPS-driven ASC formation was analyzed in the context of B cell conditional deletion of LSD1. Following LPS inoculation of hosts, LSD1-deficient B cell differentiation resulted in a two-fold reduction of splenic plasmablasts and serum IgM. LSD1-deficient plasmablasts exhibited derepression and superinduction of genes involved in immune system processes including *Siglecg*, *Sell*, *Cd86*, *Hck*, *Il10ra*, *Cd28*, *Ifitm3*, and *Amigo2*. A subset of derepressed genes were BLIMP-1 target repressed genes. Cell cycle genes were globally downregulated without LSD1, which corresponded to a decrease in the proliferative capacity of LSD1-deficient activated B cells. Plasmablasts lacking LSD1 displayed increased histone H3 lysine 4 monomethylation and chromatin accessibility at naïve B cell active enhancers and the binding sites of transcription factors BLIMP-1, PU.1, and IRF4 that mapped to LSD1 repressed genes. Together these data show that LSD1 is required for normal *in vivo* ASC formation, distinguish LSD1 as a key transcriptional rheostat in ASCs, identify a factor responsible for decommissioning naïve B cell active enhancers, and suggest a functional interaction between LSD1 and BLIMP-1, PU.1, and IRF4.

Introduction

Humoral immunity against pathogens is achieved through the function of antibody-secreting cells (ASC). In response to antigen, the ASC compartment is generated from the differentiation of naive B cells (nB) and is populated by short-lived mitotically active plasmablasts (PB) and long-lived non-cycling plasma cells (PC)[287]. Depending on the antigen, nB can give rise to a variety of responses, each evolved to efficiently neutralize the target pathogen in an antigen-specific manner[288]. nB interactions with T cell-dependent (TD) antigens results in a two-phase response. The first phase, known as the extrafollicular response results in the generation of short-lived PB that secrete mostly IgM[289]. The second phase requires the formation of germinal centers that produce PC and memory B cells. nB interactions with T cell-independent (TI) antigens, such as bacterial lipopolysaccharide (LPS), primarily results in the rapid generation of PB through an extrafollicular response[281].

As nB differentiate to ASC, they undergo widespread changes in gene expression mediated by transcription factors such as Blimp-1, XBP-1, IRF4, and PU.1[287,290]. To support the demand of constant, substantial antibody production, ASC upregulate the expression of genes that function in metabolic processes[291], as well as protein production, modification, and trafficking[292]. Transcriptional changes in ASC are accompanied by changes in the epigenome. For example, in response to LPS, global and specific increases in gene expression occur in the newly formed PB that is accompanied by alterations in chromatin accessibility at enhancers[293] and a reciprocal decrease in DNA methylation[294]. PB also exhibit alterations in H3K4me2, H3K4me3, H3K9ac, H3K27me3, and chromatin accessibility at Blimp-1 binding sites[295]. Within the PRC2 complex, the histone

methyltransferase EZH2 is critical for PB formation through H3K27me3-linked repression of transcription factor networks[296]. However, the degree to which other epigenetic modifying enzymes regulate ASC differentiation and how they influence promoter and enhancer chromatin throughout this process remains poorly understood.

Lysine-specific demethylase 1 (LSD1) is a monoamine oxidase that demethylates H3K4me1, H3K4me2, H3K9me1, and H3K9me2 through an FAD-dependent amine oxidation mechanism[11,15]. The protein structure of LSD1 consists of an enzymatically active amine oxidase-like domain, as well as SWIRM and Tower domains that facilitate protein-protein interactions[3]. By interacting with lineage-specific chromatin modifying complexes, LSD1 regulates multiple cellular differentiation pathways, including embryonic stem cell differentiation[64], neurogenesis[97], and hematopoiesis[79]. Known complexes in which LSD1 functions as a co-activator or co-repressor include those containing CoREST[36], HDAC1/2[36], the androgen receptor[15], and the estrogen receptor[297]. Importantly, LSD1 is the only histone demethylase proven to decommission enhancers during cellular differentiation by demethylating the active enhancer modification H3K4me1[64]. In the context of plasma cell differentiation, LSD1 has been shown to interact with Blimp-1[298]. The extent to which LSD1 regulates transcriptional and epigenetic changes that occur during B cell differentiation has not been determined.

Here, LSD1 expression was found to specifically increase as PB form during B cell differentiation, indicating a potential role for this protein during the process. Conditional genetic deletion of *Lsd1* in mice was used to examine its function in PB formation. LSD1 was necessary for normal LPS-induced differentiation of nB into $CD138^{+}$ PB. LSD1 repressed genes were involved in immune system processes, including Blimp-1 target

genes. Cellular proliferation was also impaired in LSD1-deficient B cells and this was coupled to reduced expression of cell cycle genes. Mechanistically, LSD1 reduced local chromatin accessibility at naïve B cell active enhancers and PU.1, IRF4, and Blimp-1 target binding sites. Without LSD1, H3K4me1 accumulated at enhancers of LSD1-regulated genes, supporting a role for this epigenetic factor in modulating gene expression. Cumulatively, this study shows that LSD1 is required for normal B cell proliferation and differentiation and functions as a transcriptional rheostat and epigenetic modifier throughout this process.

H3K4 methylation is remodeled during B cell differentiation

H3K4me2 is broadly associated with enhancers, promoters, and gene bodies and is correlated with transcription in multiple cell types and organisms, including mammalian immune system cells[64]. The dynamics of H3K4 methylation in B cell differentiation was examined by comparing the changes in H3K4me2 enrichment between nB[299] and PB derived from an ex vivo LPS differentiation model (**Fig. 2-1 A**). The comparison revealed that in PB, 6,209 genomic regions gained (red, I) while 6,592 regions lost H3K4me2 (blue, II), indicating that H3K4me2 is remodeled throughout B cell differentiation. To examine the relationship between the changes in H3K4me2 and gene expression, regions that gained and lost H3K4me2 were mapped to within 20 kb of all differentially expressed genes (DEG) between nB vs. LPS-induced PB defined previously[294]. Comparison of the $\log_2$ fold changes of the DEG mapping to regions that gained or lost H3K4me2 modifications in PB indicated that changes in gene expression were positively associated with changes in

H3K4me2 (**Fig. 2-1 B**) and suggests that this mark is remodeled and correlated with transcription during PB formation.

To understand how H3K4me2 was remodeled, the expression of known H3K4 demethylases was analyzed from data derived from discrete B cell divisions during differentiation to PB *in vivo* in response to LPS[294]. *Kdm1a* and *Kdm5c*, encoding the H3K4 histone demethylases LSD1 and JARID1C, respectively, were progressively upregulated throughout the differentiation process (**Fig. 2-1 C**). Other members of the family were not induced or expressed at appreciable levels in dividing B cells or PB. LSD1 was chosen for further investigation due to its known interaction with key ASC regulatory transcription factor Blimp-1[298] and for its ability to decommission enhancers by catalyzing H3K4me2/me1 to an unmethylated H3K4 ground state[64].

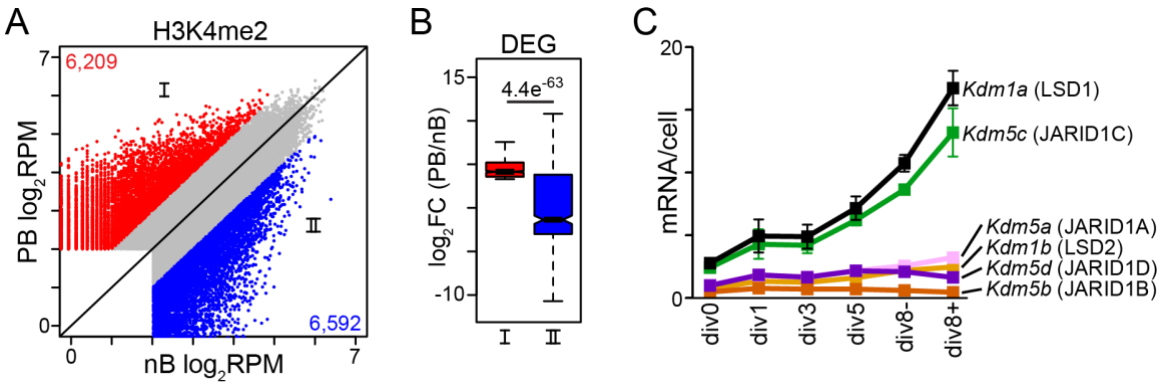


Figure 2-1: H3K4me2 is remodeled throughout B cell differentiation.
Scatter plot of genomic regions significantly enriched for H3K4me2 in naïve B cells and LPS-induced plasmablasts (37,887 total peaks). Regions that gain or lose H3K4me2 in PB by a $\log_2$ fold change of at least one are red and blue, respectively. **(B)** Box plots of the $\log_2$ fold change of the expression of genes differentially expressed from naïve B cells vs. LPS-induced plasmablasts that map within 20 kB of at least one H3K4me2 peak within PB up regions (red box, I) and PB down regions (blue box, II). Significance determined by Wilcoxon rank sum test. **(C)** Expression in mRNA/cell per division of known H3K4me2 demethylases[294]. Error bars represent mean ± SD.

LSD1 is required for normal plasmablast formation

To examine the role of LSD1 during B cell differentiation, a floxed *Lsd1* mutant allele[68] was bred onto the *Cd19*$^{Cre/+}$ background[300] to generate a B cell specific conditional knockout (CKO). Efficient *Lsd1* deletion in FACS isolated CKO splenic nB and CD138^{+} PB was observed (**Fig. 2-2 A**), and *Lsd1* mRNA levels were decreased significantly in CKO nB and PB populations (**Fig. 2-2 B**).

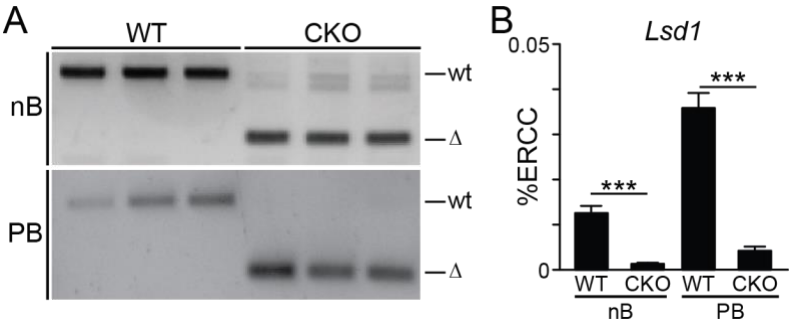


Figure 2-2 – *Lsd1* is efficiently deleted by CD19-cre.
(A) PCR on genomic DNA from WT and CKO naïve B cells (nB) and LPS-generated plasmablasts (PB) using primers spanning the floxed exon six region of *Lsd1*. **(B)** qRT-PCR on cDNA from the cell types from (A) using primers specific for floxed exon six of *Lsd1* normalized to the percentage of External RNA Controls Consortium (ERCC) spike-in RNA. Error bars represent mean ± SD. Significance determined by Student's two-tailed t-test. ***P<0.001.

Splenic B cells from naive CKO mice and *Cd19*$^{Cre/+}$ control mice (CreWT) were purified and cultured *ex vivo* in the presence of LPS, IL-2, and IL-5[301] to induce differentiation. Flow cytometry performed at day three showed that CKO B cell cultures exhibited a significant reduction in the frequency and total number of CD138^{+} PB (**Fig. 2-3 A**). Secreted antibody measured by ELISA from the same cultures showed a significant reduction in secreted IgM in CKO as compared to CreWT (**Fig. 2-3 B**). The consequence of *in vivo Lsd1* deletion on B cell differentiation was assessed by inoculating CKO and

CreWT mice with LPS and analyzing spleens at the peak B cell response time point of three days[302]. Compared to CreWT mice, CKO mice exhibited a significant reduction in the frequency and total number of $CD138^+$ PB, as well as a significant reduction in serum IgM titres (**Fig. 2-3 C,D**). To assess whether LSD1 only functions in LPS-stimulated B cell differentiation, CKO and CreWT mice were inoculated with the A/PR8/34 (PR8) strain of influenza. At seven days post infection, mice were sacrificed and the mediastinal lymph nodes were harvested and analyzed. Flow cytometry showed a significant reduction in PB formed by both frequency and total number in CKO mice (**Fig. 2-3 E**). ELISA showed a significant reduction in IgM titers in the serum (**Fig. 2-3 F**). These data indicate that LSD1 is required for normal B cell differentiation.

The intrinsic nature of the defect was examined by purifying and adoptively transferring congenically marked CKO (CD45.1) and CreWT (CD45.1/2) splenic B cells in a 1:1 ratio into μMT (CD45.2) host mice, which lack B cells[303]. Hosts were inoculated with LPS one day after transfer, and spleens were harvested and analyzed at day three. Compared to the CreWT B cell compartment, the CKO B cell compartment exhibited a significant reduction in the frequency and total number of $CD138^+$ PB, respectively (**Fig. 2-4 A**). These data show that the requirement of LSD1 for normal B cell differentiation is intrinsic to the adoptively transferred B cells.

The above results were further supported by breeding *Lsd1* floxed alleles to the tamoxifen-inducible *Rosa26*$^{CreERT2/+}$ allele[304] (IKO).). Efficient *Lsd1* deletion in IKO splenic naïve B cells was achieved compared to WT cells after tamoxifen treatment (**Fig. 2-4 B**). Purified splenic B cells from naïve IKO mice and WT mice following tamoxifen treatment were cultured *ex vivo* as above. Similar to the CKO B cells, IKO B cells

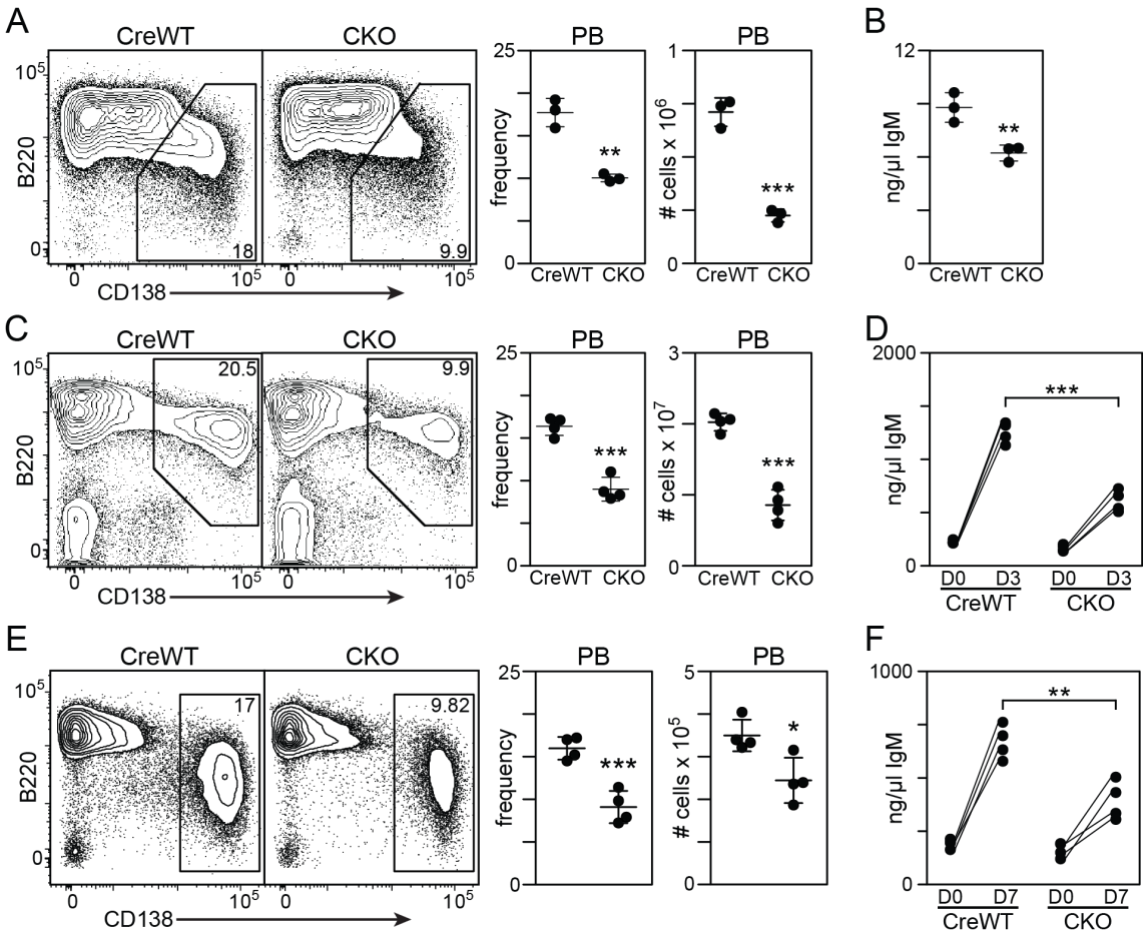


Figure 2-3 – LSD1 is required for plasmablast formation
(A) Flow cytometry analysis of B220 and CD138 expression in *ex vivo* differentiated CreWT and CKO splenic B cells cultures (left) and quantification of $CD138^+$ PB (right). **(B)** IgM media titre of B cell cultures from (A). **(C)** B220 and CD138 expression in CreWT and CKO splenocytes on day three after LPS inoculation (left) and quantification of $CD138^+$ PB (right). **(D)** IgM serum titre of mice from (C) directly before (D0) and on day three after LPS inoculation (D3). **(E)** B220 and CD138 expression in CreWT and CKO lymph node cells on day seven after PR8 influenza immunization (left) and quantification of $CD138^+$ PB (right). **(F)** IgM serum titer of mice from (E) before (D0) and after (D7) PR8 influenza immunization. All data are representative of at least two independent experiments using three to five mice per group. Error bars represent mean $\pm$ SD. Significance determined by Student's two-tailed t-test. *P<0.05, **P<0.01, ***P<0.001.

exhibited a significant reduction in PB formation and secreted IgM as compared to their respective controls (**Fig. 2-4 C, D**). Because the IKO system deletes *Lsd1* from all cells within the mouse, the effect of *Lsd1* deletion on B cells was tested by purifying splenic B

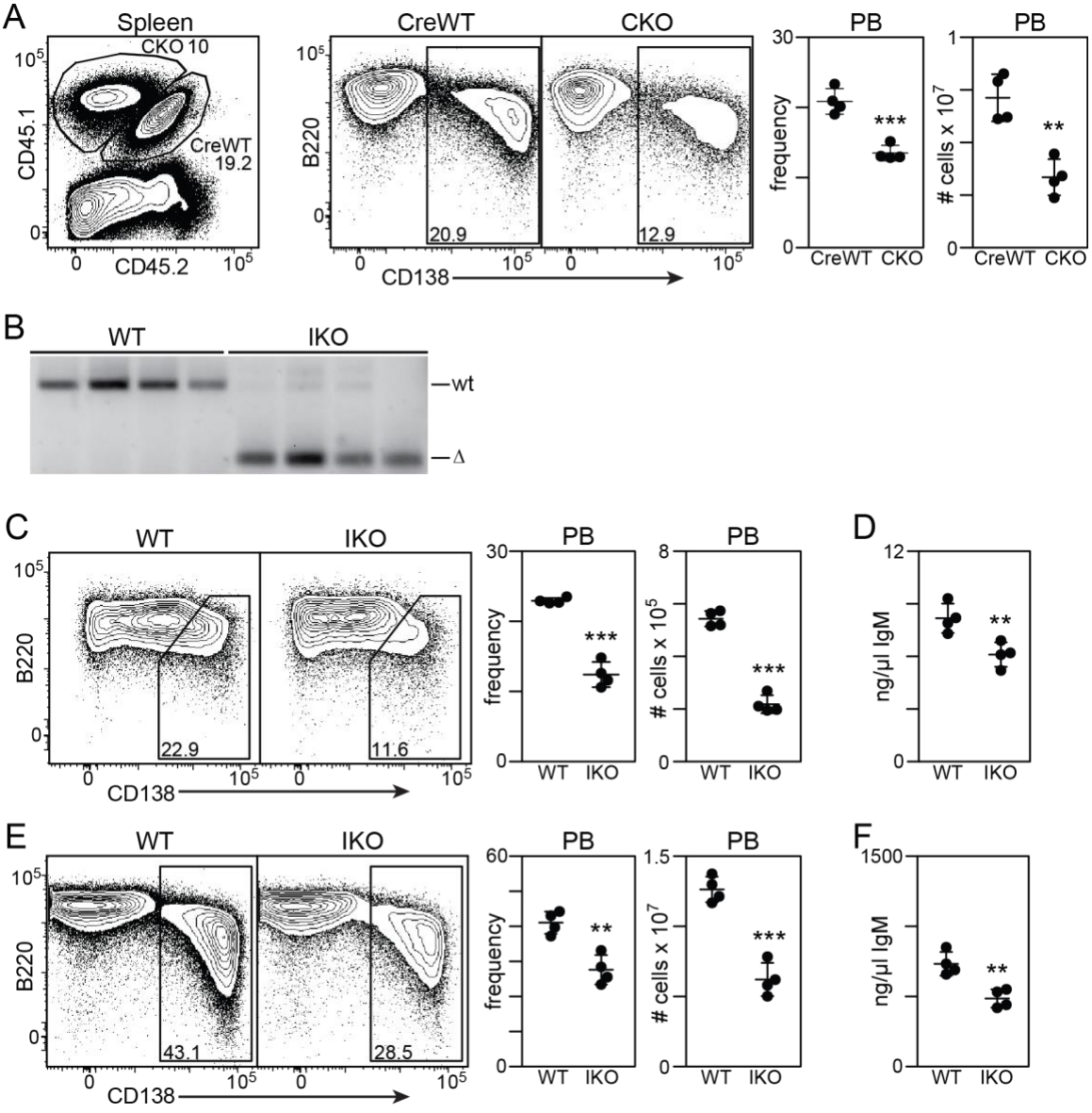
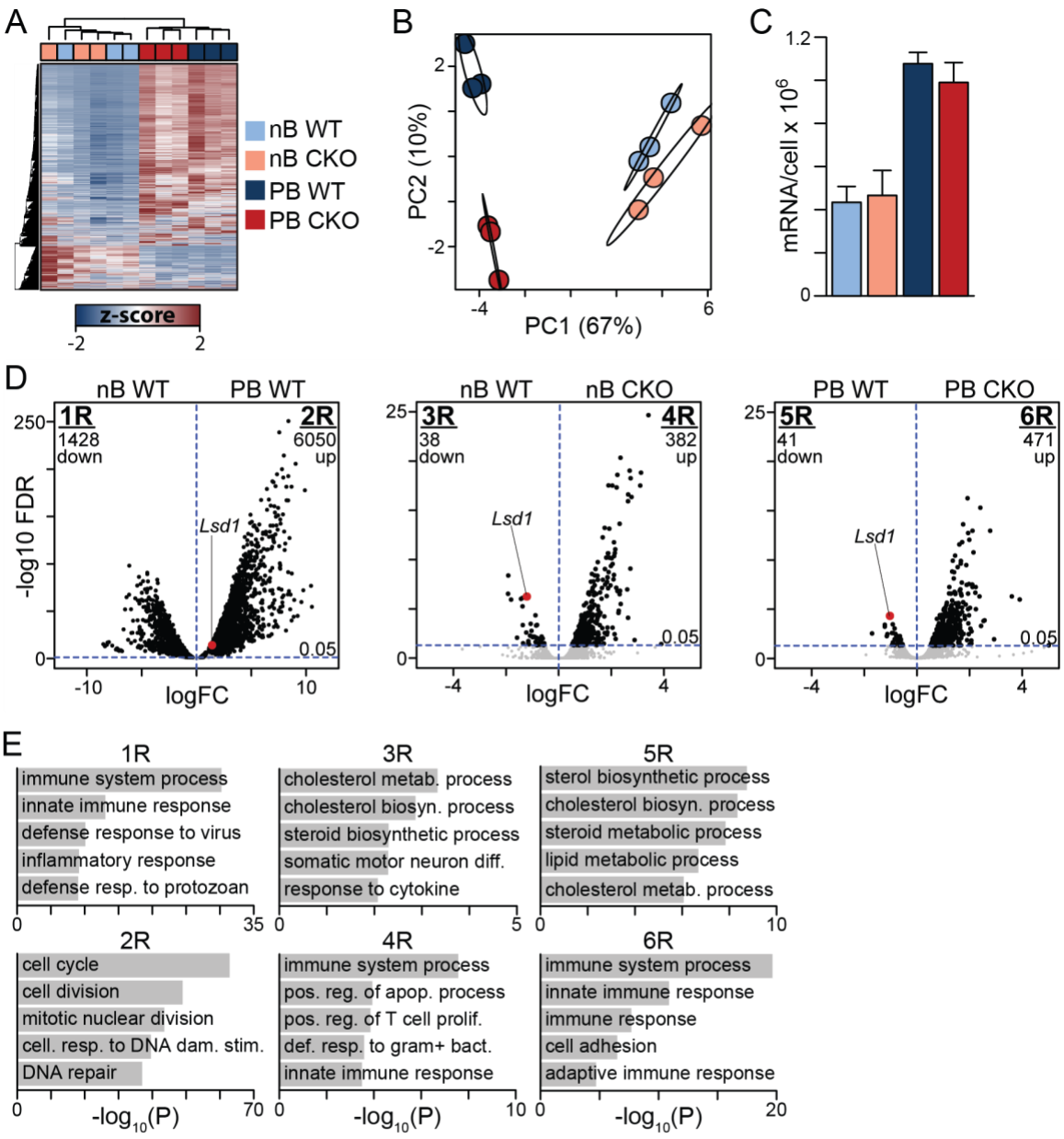


Figure 2-3 – LSD1 is intrinsically required for plasmablast formation
(A) B220 and CD138 expression in adoptively transferred CreWT and CKO splenocytes on day three after LPS inoculation (left) and quantification of $CD138^+$ PB (right). **(B)** PCR on genomic DNA from WT and IKO splenic naïve B cells using primers from Fig. 2-1 A. **(C)** B220 and CD138 expression in *ex vivo* differentiated WT and IKO splenic B cells cultures (left) and quantification of $CD138^+$ PB (right). **(D)** IgM media titre of B cell cultures from (F). **(E)** B220 and CD138 expression in adoptively transferred WT and IKO splenocytes on day three after LPS inoculation (left) and quantification of $CD138^+$ PB (right). **(F)** IgM serum titre of mice from (H) on day three after LPS inoculation. All data are representative of at least two independent experiments using three to five mice per group. Error bars represent mean ± SD. Significance determined by Student's two-tailed t-test. **P<0.01, ***P<0.001.

cells from tamoxifen-treated IKO and WT mice and transferring them separately into μMT host mice. Following LPS inoculation and analysis as above, host mice that received IKO B cells again exhibited a significant reduction in the frequency and total number of PB, respectively (**Fig. 2-4 E**). ELISA was performed on host serum and revealed that IKO cell recipient hosts had a significant reduction in serum IgM titres (**Fig. 2-4 F**). Thus, ablation of LSD1 using multiple mechanisms of genetic deletion demonstrated that B cell differentiation and humoral immune responses are impaired in the absence of LSD1 and this defect is cell-intrinsic.

LSD1 regulates the plasmablast transcriptional program

To elucidate the molecular program altered by LSD1 deficiency during PB formation, RNA-seq was performed on FACS isolated $B220^{+}GL7^{-}CD138^{-}$ nB and $CD138^{+}$ PB from both CKO and *Lsd1*$^{fl/fl}$ (WT) splenocytes three days after LPS inoculation. Hierarchical clustering and principal component analysis (PCA) of all expressed genes showed that samples stratified by both cell type and *Lsd1* deletion status, indicating an LSD1-dependent effect in both nB and PB (**Fig. 2-5 A, B**). Calculations of total mRNA per cell showed the expected increase in mRNA levels from nB to PB[294] but no difference between CKO and WT samples, indicating that *Lsd1* deletion did not affect global cellular mRNA levels. (**Fig. 2-5 C**). In the wild-type setting, 1,428 genes were downregulated and 6,050 genes were upregulated in PB as compared to nB (**Fig. 2-5 D**, referred to hereafter as DEG groups 1R and 2R, respectively). Comparison between WT and CKO nB found 38 downregulated and 382 upregulated genes in CKO nB (DEG groups 3R and 4R, respectively). Likewise, comparison between CKO and WT PB identified 41



F

Gene sets upregulated in CKO PB

Hallmark Gene Set	FDR
KRAS Signaling Up	<0.001
Inflammatory Response	<0.001
Coagulation	<0.001
IL-2 STAT5 Signaling	<0.001
Interferon Gamma Response	<0.001
Complement	<0.001

KEGG Gene Set	FDR
Cell Adhesion Molecules CAMS	<0.001
Neuroactive Ligand Receptor Interaction	<0.001
Cytokine-Cytokine Receptor Interaction	0.001
Complement and Coagulation Cascades	<0.001
Arrhythmogenic Right Ventricular Cardiomyopathy	0.001
Hematopoietic Cell Lineage	0.001

Figure 2-5 – LSD1 regulates the plasmablast transcriptional program.
(A) Heatmap of hierarchical clustered, z-score normalized expression data (mRNA/cell) of all 11,909 detected genes between the indicated groups. **(B)** Top two principle components

(PC1, PC2) from PCA of z-score normalized mRNA/cell expression of genes from (A). Circles represent 99% confidence intervals. **(C)** Average mRNA per cell per sample group. **(D)** Volcano plots of differentially expressed genes (DEG) between the indicated comparison. Plotted are -log10(FDR) and logFC based off of differential expression analysis of absolute changes in gene expression. LSD1 is indicated in each plot (red dot). **(E)** Top five most significant gene ontologies for each DEG group from Figure 3D. **(F)** Tables indicating top six Hallmark and KEGG gene sets that are significantly enriched (FDR < 0.01) in genes upregulated in CKO PB through GSEA.

downregulated and 471 upregulated genes in CKO PB (DEG groups 5R and 6R, respectively). These data show that LSD1 predominantly functions as a transcriptional repressor in this system and identify genes that are dysregulated in its absence.

The global functions of LSD1-dysregulated genes were investigated by performing Gene Ontology (GO) analysis on DEG groups (**Fig. 2-5 E**). The top enriched GO term for 6R DEG was immune system process. This was further supported by gene set enrichment analysis (GSEA) of the WT and CKO PB RNA-seq data comparisons to all HALLMARK and KEGG gene sets (**Fig. 2-5 F**), which revealed that genes involved in processes such as the inflammatory response, cell signaling, complement cascade, coagulation, cellular adhesion, and cytokine-cytokine receptor interactions were upregulated in the absence of LSD1. For example, LSD1-deficient PB upregulated the pro-inflammatory genes *C1qa*, *C1qb*, *C1qc*, *C3*, *C4bp*, *Cd14*, and *Tlr7*, as well as the IFN-γ response genes *Ifit2*, *Ifitm3*, *Il10ra*, *Usp18*, *Vamp5*, and *Vcam1*. These data therefore show that LSD1 normally represses pro-inflammatory signals during B cell differentiation.

Interestingly, the top enriched GO term (immune system process) for genes upregulated in CKO PB compared to WT controls (1R) matched the top GO term for genes downregulated in WT PB compared to WT nB (6R). This implies that genes downregulated when cells normally differentiate from nB to PB were derepressed in the

absence of LSD1. This was also supported by the finding that between DEG groups 1R (normally repressed in PB) and 6R (upregulated in CKO PB) there were 143 genes that overlapped, which was 2.5-fold more than expected by chance (**Fig. 2-6 A**). No significant overlap was observed between 1R and 5R (**Fig. 2-6 A**). To further examine the derepressed genes, genes were ranked by expression differences between WT and CKO PB and compared to the 200 most significant DEG from 1R (genes repressed in PB) using GSEA (**Fig. 2-6 B**). This analysis showed that genes aberrantly upregulated in CKO PB were significantly enriched for genes normally repressed in WT PB, further underscoring the importance of LSD1 in gene repression during PB formation. Additional analyses using gene sets defining follicular splenic B cells and plasma cells[292] corroborated the above GSEA results (**Fig. 2-6 C**). Example genes that exhibited this expression pattern included those that function in complement activation (*Cfp*[305]), homing (*Itgb7*[306], *Sell*[307]), response to bacteria (*Mpeg1*[308]), signaling (*Lsp1*[309], *Plaur*[310], *Siglecg*[311]), and survival (*Gimap4*[312]; **Fig. 2-6 D**).

GSEA performed with the top 200 up DEG from group 2R (normally upregulated in PB) revealed that some genes in WT PB were superinduced in the absence of LSD1 (**Fig. 2-6 E**). Example genes that exhibited this expression pattern included *Ly6c*, which encodes a plasma cell surface marker[313], and those that function in adhesion (*Amigo2*[314]), homing (*Cd68*[315]), proliferation (*Cd300a*[316], *Uchl1*[317]), response to virus (*Ifitm3*[318]), and signaling (*Cd28*[319], *Dusp14*[320]; Fig. 2-6 H). These results highlight LSD1 as a transcriptional rheostat throughout B cell differentiation.

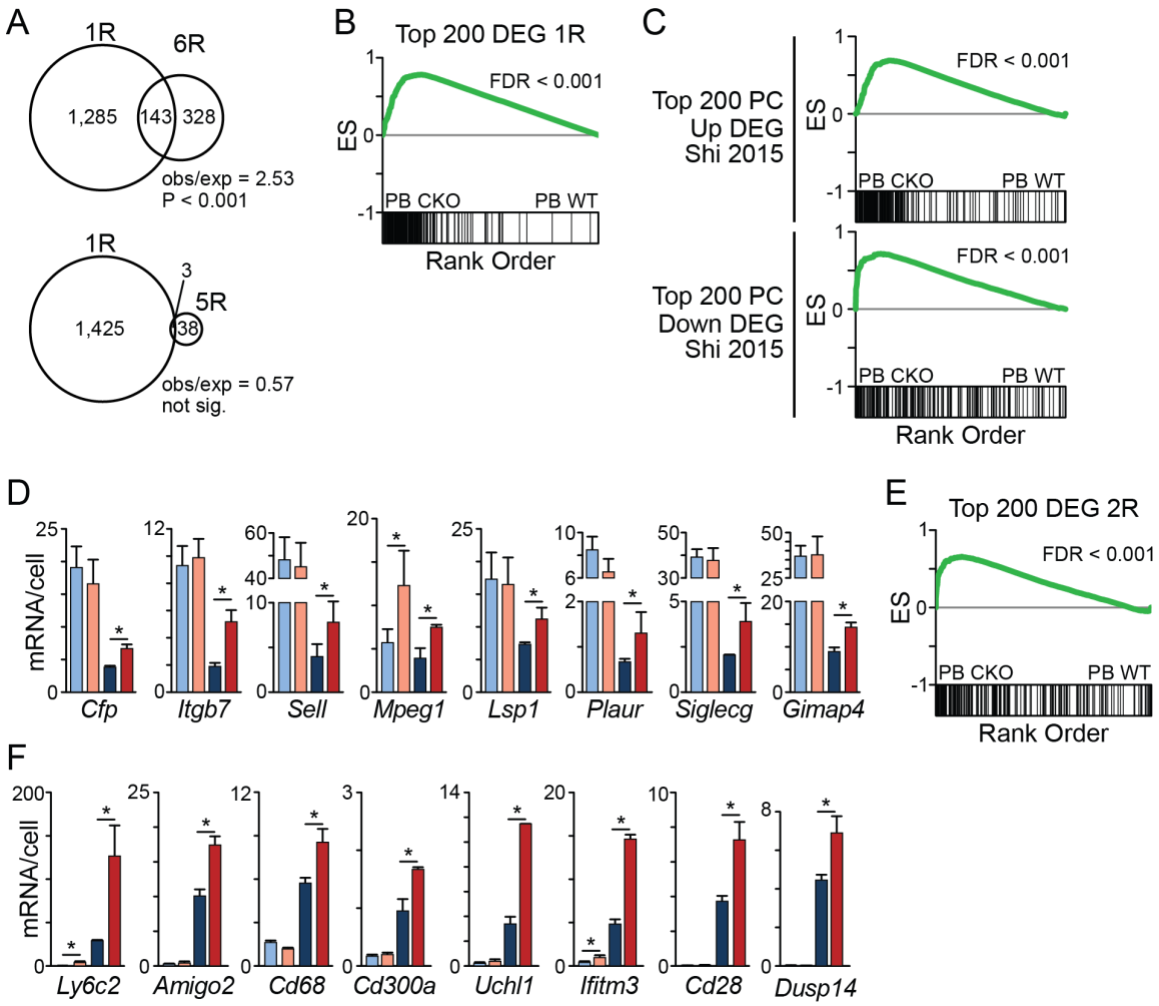


Figure 2-6 – LSD1 deletion results in gene derepression and gene superinduction
(A) Overlapping DEG between the indicated comparisons. The DEG groups 1R, 5R, and 6R are from Fig. 2-5 D. obs/exp refers to the ratio of observed DEG overlap over expected overlap according to a permutation test. **(B**) GSEA analysis of PB WT and PB CKO using a gene set of the top 200 most significant 1R DEG. **(C)** GSEA analysis for enrichment of the top 200 most significant up and down DEG between splenic follicular B cell and plasma cell samples from Shi et al. (2015) in the ranked gene list from Figures 3E and 3G. **(D)** DEG exhibiting derepression in PB. **(E)** GSEA analysis of PB WT and PB CKO using a gene set of the top 200 most significant 2R DEG. **(F)** DEG exhibiting superinduction. Error bars represent mean ± SD. Significance determined by edgeR. **FDR*<0.05.

Blimp-1 target repressed genes are regulated by LSD1

One possible explanation for the transcriptional dysregulation observed in CKO PB is LSD1-dependent dysregulation of essential transcription factors. However, this did not

appear to be the case as genes encoding nB transcription factors (BACH2, BCL6, ETS1, IRF8, PAX5, SPIB) and PB transcription factors (Blimp-1, IRF4, XBP-1) were appropriately expressed and regulated (**Fig. 2-7 A**). However, Blimp-1 has been shown to recruit histone modifying complexes to facilitate gene repression[295] and can physically interact with LSD1[298]. To determine if direct Blimp-1 target genes were dysregulated when LSD1 was deleted, Blimp-1 activated and repressed gene sets[295] were tested for enrichment in ranked gene lists derived from the comparisons nB WT vs. PC WT; nB WT vs. nB CKO; and PB WT vs. PB CKO (**Fig. 2-7 B**). As expected in the wild-type setting, the GSEA of WT nB and PB identified genes up and down regulated by Blimp-1 (**Fig. 2-7 B**, top). No enrichment involving Blimp-1 target genes was observed when comparing WT and CKO nB (**Fig. 2-7 B**, middle). In contrast, when comparing WT and CKO PB, Blimp-1 repressed target genes failed to be fully downregulated (**Fig. 2-7 B**, bottom), suggesting that LSD1 deficiency leads to derepression of Blimp-1 target genes. Examples included the genes *Mpeg1* and *Sell* as described above (**Fig. 2-6 D**), a gene encoding a glycoprotein found in neutrophil azurophilic granules with putative amidase activity (Plbd1[321]), and those involved in response to bacteria (Tlr1[322]) and signaling (Evl[323], Hck[324], Hvcn1[325], Il10ra[326]; **Fig. 2-7 C**). These data indicate that LSD1 is, in part, responsible for repressing genes inhibited by Blimp-1.

LSD1 promotes B cell proliferation

Annotation of differentially expressed genes to HALLMARK and KEGG gene sets by GSEA identified proliferation and cell cycle genes downregulated in CKO PB as compared to WT (**Fig. 2-8 A**). This is consistent with fewer PB observed following LPS-

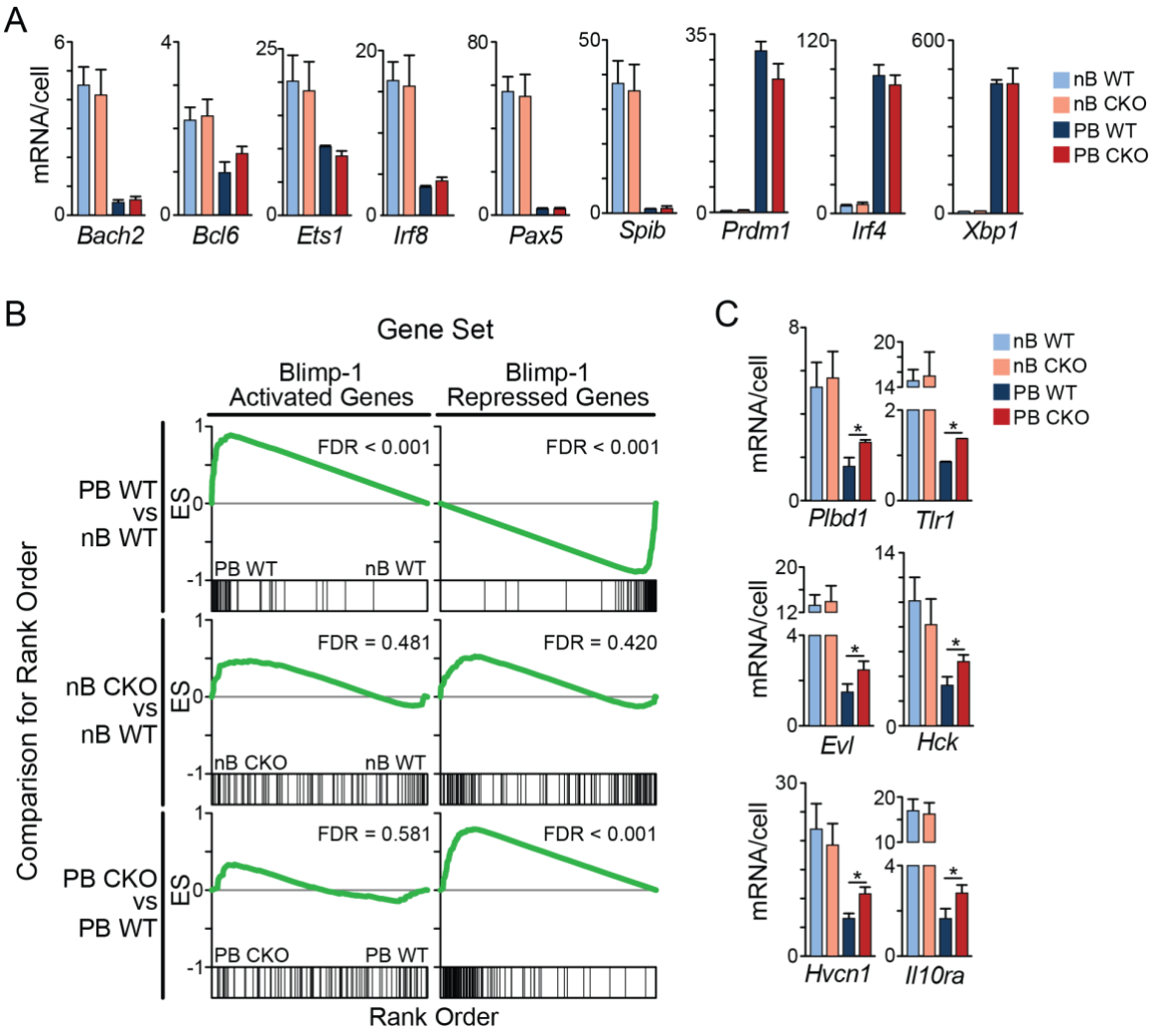


Figure 2-7 – Blimp-1 target repressed genes are regulated by LSD1
(A) RNA-seq mRNA/cell expression data of key B cell transcription factors. **(B)** GSEA analysis for enrichment of Blimp-1 activated genes (left) and Blimp-1 repressed genes (right) in all detected genes ranked by expression difference between the indicated sample group comparisons. **(C)** Example Blimp-1 target repressed DEG. Error bars represent mean ± SD. Significance determined by edgeR. **FDR*<0.05.

mediated *in vivo* B cell differentiation, suggesting that LSD1 may regulate B cell proliferation. To test if there was a proliferation defect, the proliferative capacity of CreWT and CKO B cells in response to LPS was quantified by CTV staining of purified splenic B cells that were cultured *ex vivo* as above. Cultures were analyzed at 24, 36, 48, 60, and 72

A

Gene sets downregulated in CKO PB

Hallmark Gene Set	FDR
E2F Targets	<0.001
G2M Checkpoint	<0.001
MYC Targets V1	<0.001
DNA Repair	<0.001
MYC Targets V2	<0.001
MTORC1 Signaling	0.001
Mitotic Spindle	0.001
Cholesterol Homeostasis	0.006

KEGG Gene Set	FDR
DNA replication	<0.001
Mismatch Repair	<0.001
Homologous Recombination	<0.001
Nucleotide Excision Repair	<0.001
Base Excision Repair	<0.001
Cell Cycle	0.001
Proteasome	0.001
Aminoacyl tRNA Biosynthesis	0.005
Spliceosome	0.006

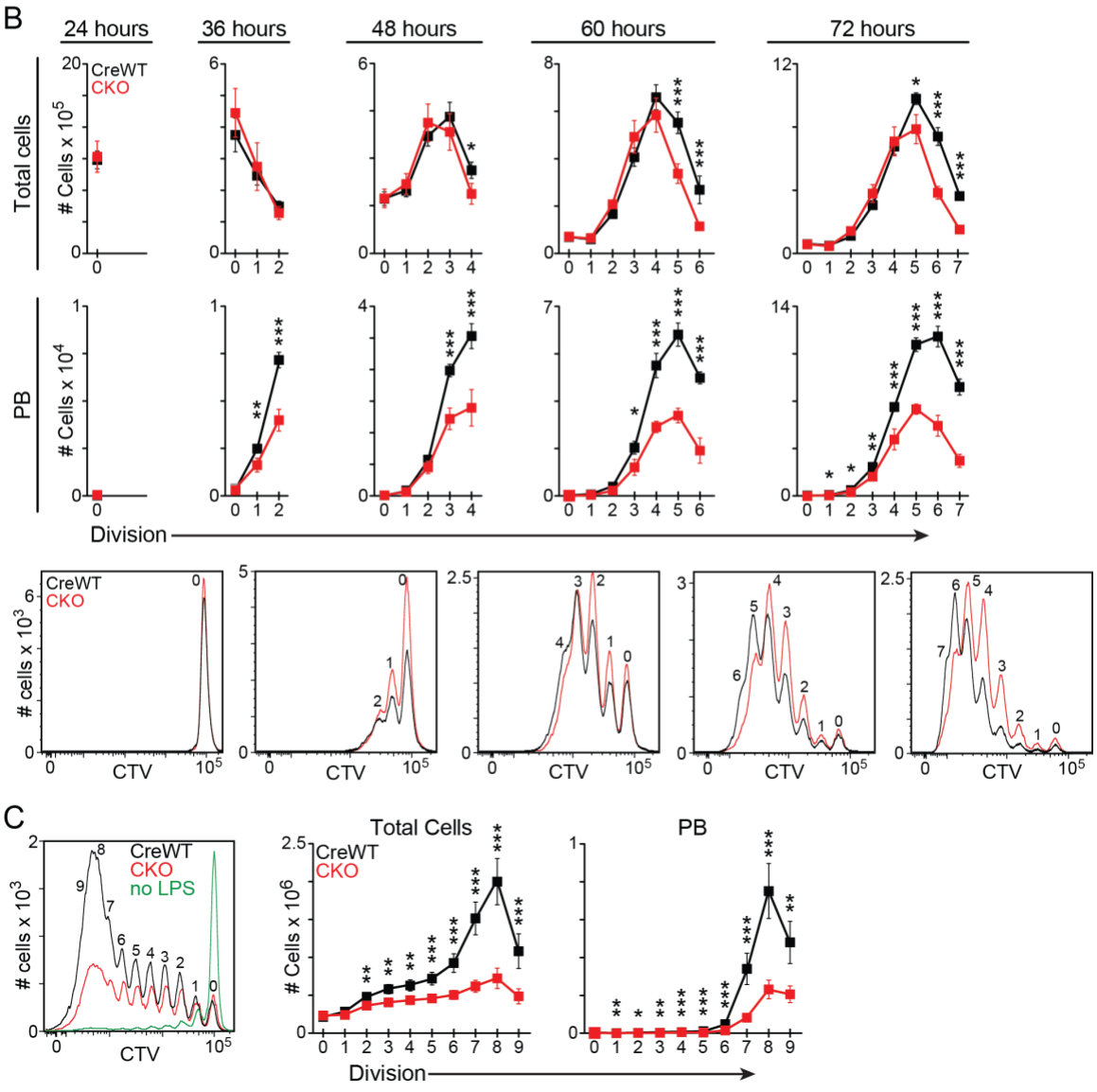


Figure 2-8 – Blimp-1 target repressed genes are regulated by LSD1

(A) Tables indicating all Hallmark and KEGG gene sets that are significantly enriched (FDR < 0.01) in genes downregulated in CKO PB through GSEA. **(B)** Total cells and total CD138^{+} PB per division of *ex vivo* differentiated CreWT and CKO splenic B cells at five

time points (top) and corresponding flow cytometry analysis of CTV (bottom). **(C)** Flow cytometry analysis of CTV in adoptively transferred CreWT and CKO splenic B cells on day three after LPS inoculation (left) and quantification of total cells and total $CD138^+$ PB per division (right). Data are representative of at least two independent experiments using three to five mice per group. Error bars represent mean ± SD. Significance determined by Student's two-tailed t-test. $*P<0.05$, $**P<0.01$, $***P<0.001$.

hours (**Fig. 2-8 B**). When assessed by division, CKO cultures produced fewer total cells after 36 hours and fewer $CD138^+$ PB at all time points after 24 hours of culture.

The *in vivo* proliferation defect was characterized by purifying CKO and CreWT B cells, staining them with CTV, and adoptively transferring them into a μMT host and inoculating them with LPS as above. CKO cells had significantly reduced cells in divisions two through nine, and the total number of $CD138^+$ PB in later divisions was decreased substantially (**Fig. 2-8 C**). Together, these data indicate that LSD1 is critical for normal B cell proliferation in response to LPS.

LSD1 regulates chromatin accessibility at ETS and IRF transcription factor motifs

ATAC-seq was performed on the same samples as RNA-seq to assess the global effects of LSD1 deficiency on active regulatory elements during B cell differentiation. PCA of ATAC-seq data showed that samples stratified by both cell type and *Lsd1* deletion status (**Fig. 2-9 A**), supporting a chromatin regulatory role for LSD1 in nB and PB. Differential accessibility analysis indicated that in the wild-type setting, PB lost 19,461 and gained 12,646 accessibility regions compared to nB (**Fig. 2-9 B**, referred to hereafter to as differentially accessible region (DAR) groups 1A and 2A, respectively). Examining WT and CKO sample comparisons of the same cell type found that both CKO nB and CKO PB underwent mostly targeted increases in chromatin accessibility (groups 4A and 6A,

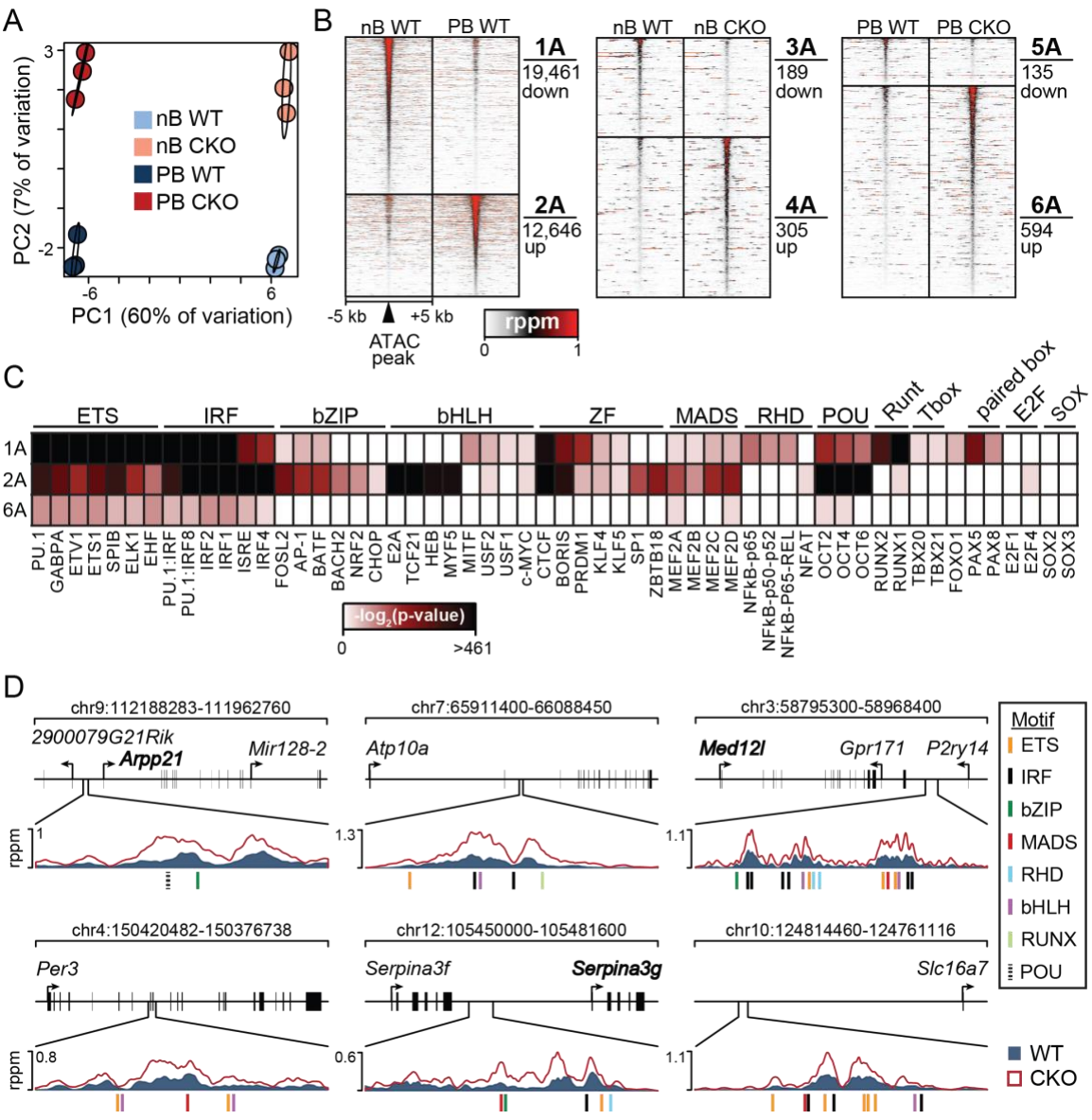


Figure 2-9 – LSD1 regulates chromatin accessibility at ETS and IRF transcription factor motifs.

(A) Top two principle components (PC1, PC2) from principle component analysis of z-score normalized rppm values of all ATAC-seq peaks (72,519 total) and sample group 99% confidence intervals (black ovals). rppm, reads per peak per million. **(B)** Heatmap depicting differentially accessible regions between the indicated comparisons. rppm +/- 5 kb around the peak is shown. **(C)** Heatmap displaying -log$_2$(p-values) for transcription factor binding motifs enriched in the indicated DAR identified through HOMER known motif analysis. **(D)** Gene tracks of example DAR mapping to a 6R gene. Transcription factor family motifs are indicated by colored dashes under each track.

respectively).

To gain insight into the transcription factors associated with LSD1-regulated chromatin in PB, motif analysis[327] was performed on DAR groups 1A, 2A, and 6A (**Fig. 2-9 C**). Group 1A DAR, which contained nB-accessible regions that normally would be inaccessible in PB, were enriched for motifs of transcription factors known to be important in nB development and maturation, including ETS1[328], RUNX1[329], and PAX5[330]. Group 2A DAR, which contained newly accessible regions in PB, were enriched for motifs of transcription factors known to be important for plasma cell formation and function, including E2A[331], OCT2[332], and IRF4[333]. Group 6A DAR, which were more accessible due to loss of LSD1, were primarily enriched for motifs of the transcription factor families ETS and IRF and were modestly enriched for motifs of the transcription factor families MADS and POU, suggesting that LSD1 functions to restrict chromatin accessibility at the binding sites of these factors. The occurrence of several motifs, including E2F1, Sox2, and Sox3 do not occur in any of the DAR groups (**Fig. 2-9 C**), suggesting specificity for the identified motifs. Example 6A DAR that contained motifs of transcription factors belonging to these families and that also mapped to a 6R DEG are displayed (**Fig. 2-9 D;** *Arpp21*, *Atp10a*, *Med12l*, *Per3*, *Serpina3g*, *Slc16a7*). Overall, these data link PB-based chromatin closure with LSD1 at sites enriched with ETS, IRF, MADS, and POU family motifs and suggests a functional relationship between LSD1 and transcription factors of these families.

LSD1 restricts chromatin accessibility at naïve B cell enhancers in plasmablasts

Analysis of DAR revealed a 2.05-fold more than expected overlap between DAR groups 6A and 1A compared to no significant overlap between groups 5A and 1A, indicating that LSD1 restricts chromatin accessibility at regions normally accessible in nB (**Fig. 2-10 A**). To determine if LSD1-specific DAR occurred at nB regulatory regions, 1A, 2A, and 6A DAR were analyzed for enrichment of the active chromatin histone modifications H3K4me1 and H3K27ac from published nB datasets[334] (**Fig. 2-10 B**). Compared to 2A, both 1A and 6A DAR were significantly enriched for both marks, suggesting that in nB, 1A and 6A DAR were located at cis-regulatory elements. The overlap of DAR and active enhancers (containing H3K4me1 and H3K27ac, but not H3K4me3[335]) in several cell types was assessed by an odds ratio (**Fig. 2-10 C**). The analysis indicated that 1A, 2A, and 6A DAR significantly overlapped active enhancers from various cell types, which is expected given that enhancers can be shared between cell types[336], but the highest degree of overlap for 1A and 6A DAR was observed with nB active enhancers. Conversely, DAR rarely occurred at active promoters (containing H3K27ac and H3K4me3, but not H3K4me1). nB active enhancers overlapping with 1A, 2A, and 6A DAR were tested for enrichment of H3K4me2 in wild-type nB and PB cells (**Fig. 2-10 D**). Both 1A and 6A nB enhancers exhibited a significant decrease in H3K4me2 whereas 2A nB enhancers exhibited a significant increase, demonstrating that 1A and 6A nB enhancer regions normally lose LSD1-target H3K4 methylation in PB. Overall, these data imply that LSD1 functions to decommission nB active enhancers.

Motif analysis was performed on 1A and 6A nB enhancers to gain insight into the transcription factors possibly bound at LSD1-regulated nB enhancers (**Fig. 2-10 E**). In both enhancer sets, the most significantly enriched motifs included ETS and IRF family

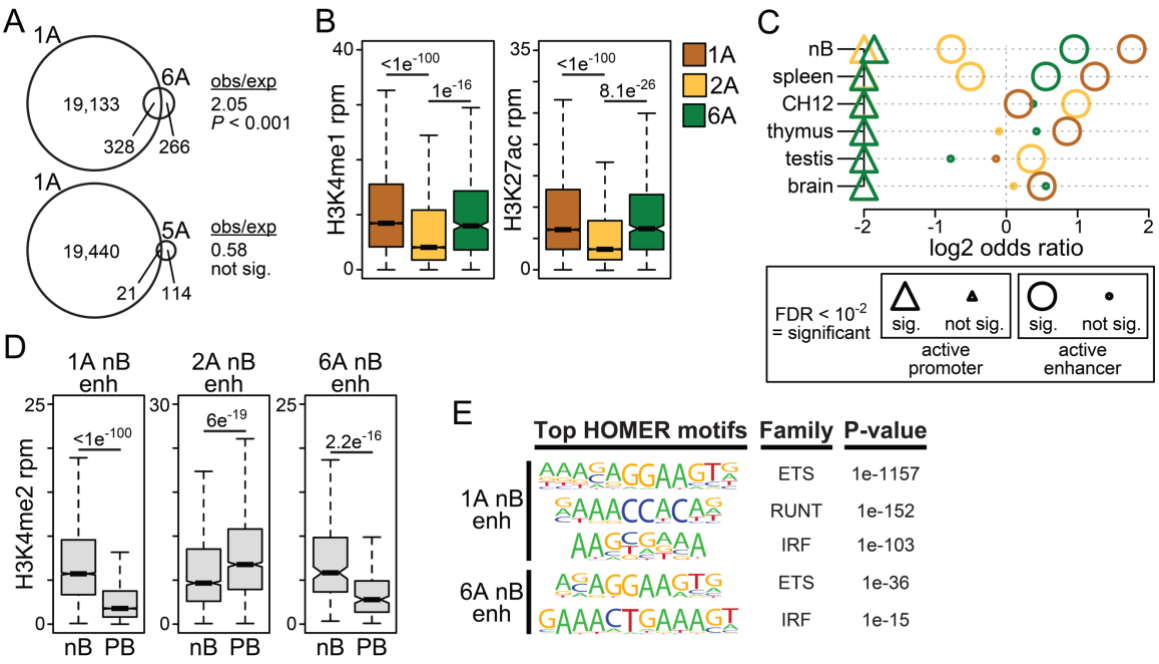


Figure 2-10 – LSD1 restricts chromatin accessibility at naïve B cell enhancers in plasmablasts

(A) Overlap between DAR group comparisons 1A vs 6A and 2A vs 6A. obs/exp refers to the ratio of observed DAR overlap over expected overlap according to a permutation test. **(B)** ChIP-seq rpm enrichment of nB H3K4me1 (left) and H3K27ac (right) for DAR groups 1A, 2A, and 6A. **(C)** Log_2 odds ratios of DAR group enrichment with active enhancers and active promoters from six different cell types. **(D)** Boxplot of ChIP-seq enrichment of nB and PB H3K4me2 for nB enhancers mapping to 1A DAR and 6A DAR. **(E)** Top significantly enriched transcription factor motifs identified through HOMER *de novo* motif analysis for 1A nB enh and 6A nB enh. Significance determined by Wilcoxon rank sum test (B), Fisher's exact test (C), or Student's two-tailed t-test (D). rpm, reads per million.

factors. These data suggest that LSD1 restricts chromatin accessibility at enhancers containing ETS and IRF motifs during PB differentiation.

The relationship between LSD1 and transcription factors was explored by analyzing published ChIP-seq data for the factors PU.1, IRF4, and Blimp-1 from *ex vivo* LPS-induced PB[295]. PU.1 was chosen because it is an ETS family transcription factor known to regulate the development and differentiation of B cells and also interact with IRF factors[290,337]. IRF4 was chosen because of its clear and critical role during B cell

differentiation[333]. All binding sites per transcription factor were analyzed for H3K4me2 enrichment in wild-type nB and PB (**Fig. 2-11 A**). All three sets of binding sites exhibited a significant decrease in H3K4me2, suggesting a role for LSD1 at these sites. Binding of the each of the above factors was found to occur within the 594 group 6A DAR (170, 17, 34, respectively), suggesting that these transcription factors bind at LSD1-regulated chromatin and potentially contribute to the recruitment of LSD1 (**Fig. 2-11 B**). These results do not preclude the action of additional factors from influencing LSD1-regulated chromatin.

The role of LSD1 at PU.1, IRF4, and Blimp-1 binding sites that map to LSD1-regulated genes were examined. To start, transcription factor binding sites were mapped to within 100 kb of genes upregulated in LSD1-deficient PB (6R DEG), resulting in three distinct groups of transcription factor binding sites (6R PU.1, 512 binding sites; 6R IRF4, 290 binding sites; 6R Blimp-1, 144 binding sites). Each group was assessed for enrichment of the LSD1-target histone modification H3K4me2 in wild-type nB and PB[295,299] (**Fig. 2-11 C**). The analysis found that H3K4me2 levels decreased in PB compared to nB for all three groups, suggesting a role for LSD1 at these sites. To explore this further, chromatin accessibility data from this study were examined similarly (**Fig. 2-11 D**). Each of the three transcription factor binding site groups exhibited a significant increase in chromatin accessibility in LSD1-deficient PB. For a negative control, regions that were bound by SOX2, a transcription factor not involved in regulating B cell differentiation[338], were analyzed as above (6R SOX2, 294 binding sites; **Fig. 2-11 E,F**). No significant differences were found. Example PU.1, IRF4, and Blimp-1 transcription factor binding sites identified in the above analyses are displayed (**Fig. 2-11 G;** *Hmgcll1*, *L3mbtl3*, *Timd2*). These data

support a chromatin remodeling role for LSD1 at PU.1, IRF4, and Blimp-1 target binding sites that map to LSD1-regulated genes.

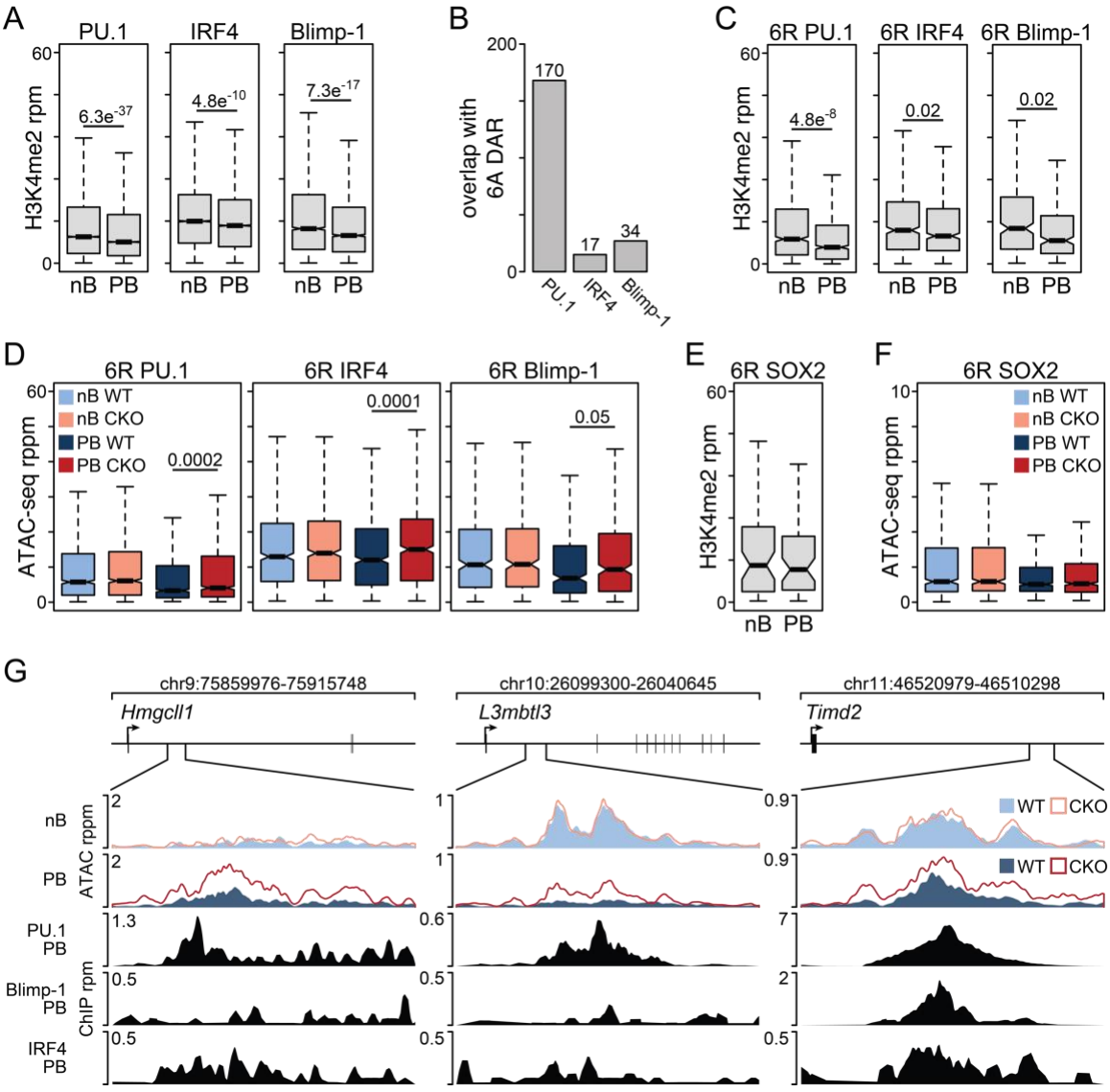


Figure 2-11 – LSD1 restricts chromatin accessibility at Blimp-1, PU.1, and IRF4 binding sites in plasmablasts

(A) ChIP-seq rpm enrichment of nB and PB H3K4me2 for all PU.1, IRF4, and Blimp-1 binding sites. **(B)** Bar plot depicting the number of overlapping 6A DAR with PU.1, IRF4, and Blimp-1 transcription factor binding sites. **(C)** Boxplot of ChIP-seq enrichment of nB and PB H3K4me2 for PU.1 binding sites, IRF4 binding sites, and Blimp-1 binding sites mapping to 6R DEG. **(D)** Boxplot of chromatin accessibility of the indicated sample groups at 6R PU.1, 6R IRF4, and 6R Blimp-1 regions. **(E, F)** Same as C and D but with

SOX2 binding sites. **(G)** Gene tracks of example transcription factor binding sites mapping to a 6R gene that exhibit significant increases in chromatin accessibility in PB CKO. Significance determined by Student's two-tailed t-test. rppm, reads per peak per million; rpm, reads per million.

Aberrant accumulation of H3K4me1 at LSD1-regulated loci

The effect that LSD1 had on chromatin accessibility during B cell differentiation was mainly restrictive and occurred at enhancer regions, implying that LSD1 demethylates the active enhancer histone modification H3K4me1 in this system. To determine if this was the case, H3K4me1 levels at LSD1-regulated DAR (**Fig. 2-9 B, group 6A**) were assayed by ChIP. Chromatin was prepared from CreWT nB, CKO nB, CreWT PB, and CKO PB and H3K4me1 ChIP-qPCR was performed on a set of nine regions previously defined (**Fig. 2-9 D, 2-11 G**). Regions mapping to the derepressed genes *Med12l*, *Slc16a7*, and *L3mbtl3* and the superinduced genes *Arpp21*, *Atp10a*, *Per3*, and *Hmgcll1* exhibited significant increases in H3K4me1 in CKO PB compared to CreWT PB (**Fig. 2-12 A**). Of these DAR, those that mapped to the genes *Arpp21*, *Atp10a*, *Hmgcll1*, and *L3mbtl3* mapped to a nB active enhancer (**Fig. 2-10 C**).

To further explore the role of LSD1 in regulating H3K4me1, twelve additional potential enhancer regions mapping to LSD1-regulated genes (**Fig. 2-6 D,F, 2-7 C**) were chosen based on 1) transcription factor binding of PU.1, Blimp-1, or IRF4; and 2) presence of H3K4me1 in nB reported previously[295,334]. Regions mapping to the derepressed genes *Hck*, *Sell*, and *Siglecg*, and the superinduced genes *Amigo2*, *Cd28*, and *Ifitm3* exhibited significant increases in H3K4me1 in PB in the absence of LSD1 (**Fig. 2-12 B**). Five out of six regions, including all three derepressed gene genes, mapped to a nB active enhancer

(**Fig. 2-13**). Other regions did not reach statistical significance for increases in H3K4me1 in the absence of LSD1, suggesting that the role of LSD1 is specific to certain regions.

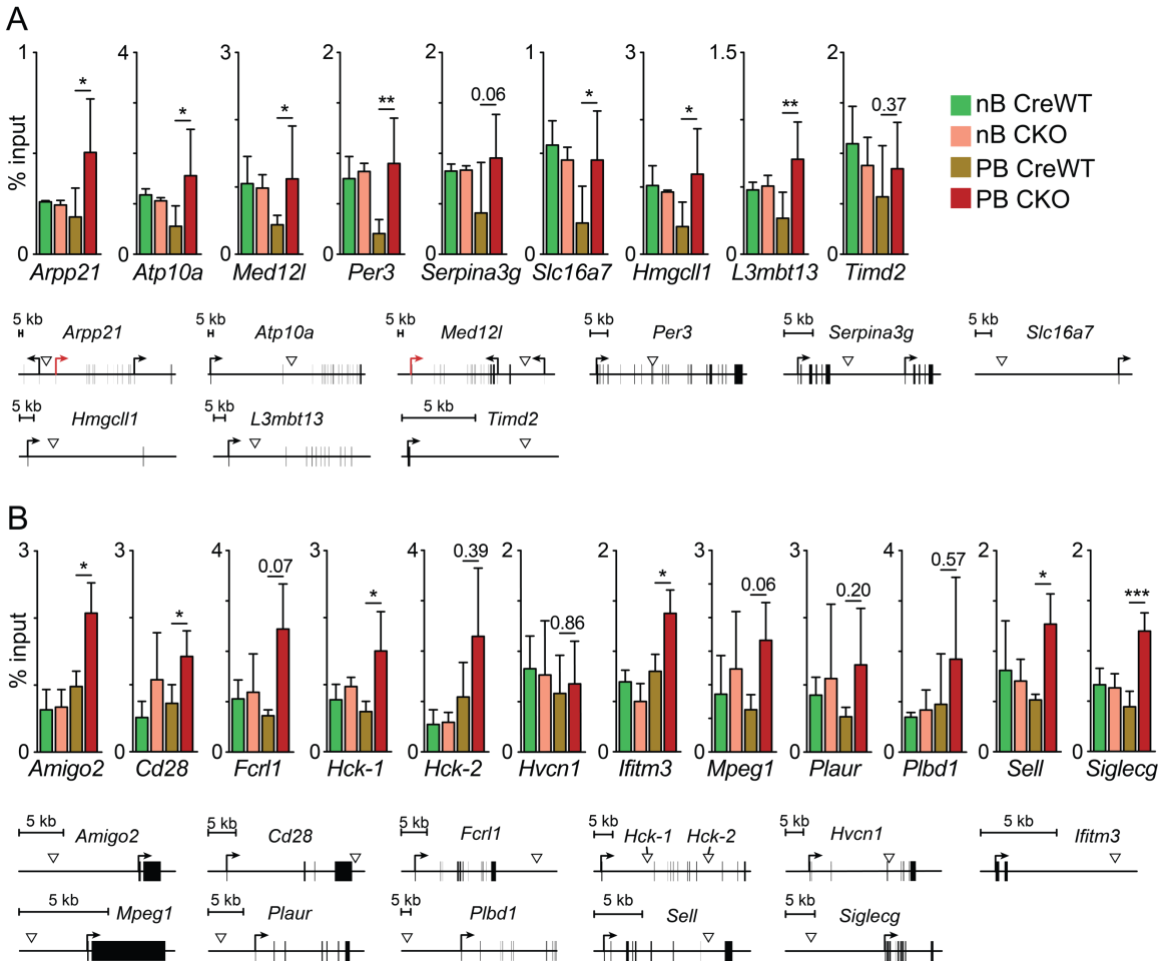


Figure 2-12 – Aberrant accumulation of H3K4me1 at LSD1-regulated loci.
(A, B) ChIP-qPCR for H3K4me1 enrichment displayed as % of input at the indicated genomic regions. Data are combined from two independent experiments using three mice per group. Primer location relative to the gene is shown below bar plots. Error bars represent mean ± SD. Significance determined by Student's two-tailed t-test. **P*<0.05, ***P*<0.01, ****P*<0.001.

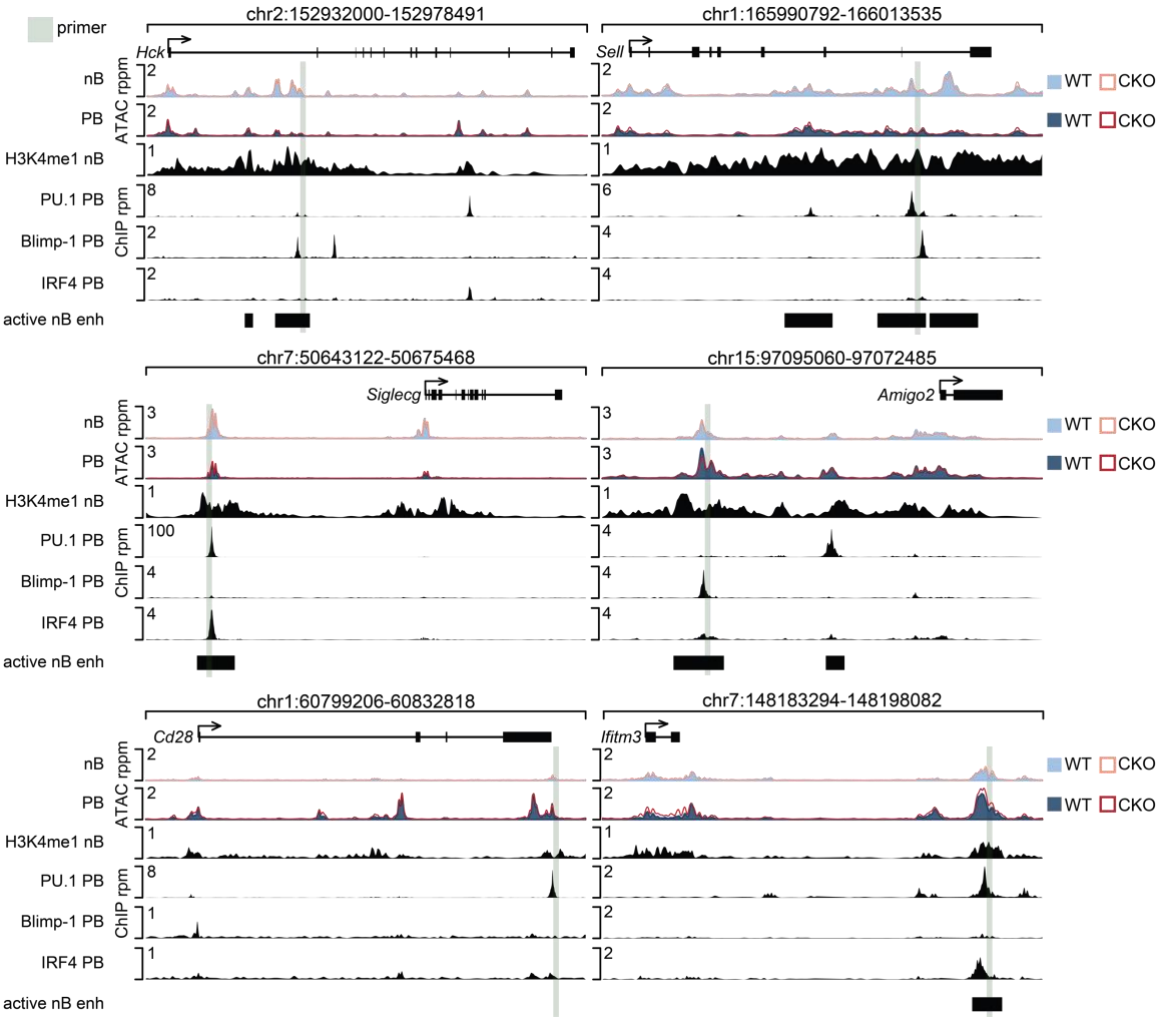


Figure 2-13 – Gene tracts of LSD1-repressed genes.
Gene tracks corresponding to the genes in Figure 8B displaying significant differences in H3K4me1. Data depicted include ATAC-seq data from this study, nB ChIP-seq data for H3K4me1[334], and ChIP-seq data for the transcription factors PU.1, Blimp-1, and IRF4[295]. Active nB enhancers are depicted as black horizontal bars. The regions probed with primers for ChIP-qPCR are demarcated with grey vertical bars. rppm, reads per peak per million; rpm, reads per million.

Some of the regions significant for H3K4me1 increases were further examined in the context of other model systems. Using *in vitro*-derived effector $CD8^+$ T cell ChIP-seq data of the enhancer modifications H3K4me1 and H3K27ac, as well as IRF4 binding[339], four regions were identified as IRF4-bound enhancers (**Fig. 2-14 A**). *Ifitm3* and *L3mbtl3* were identified as responsive to changes in IRF4 expression in $IRF4^{-/+}$ exhausted $CD8^+$ T cells[340]. *Atp10a* and *Siglecg* were identified as being bound by BATF and NFAT in addition to IRF4 and represent chronic infection signature genes [340]. Using ATAC-seq data and PU.1 and LSD1 ChIP-seq data from mouse-engrafted MLL-AF9 primary acute myeloid leukemia cells either treated or not treated with an LSD1 inhibitor[341], regions mapping to *Serpina3g*, *Ifitm3*, *L3mbtl3*, and *Siglecg* were identified as being bound by LSD1, PU.1, and exhibiting increased accessibility upon pharmacological inhibition of LSD1 (**Fig. 2-14 B**). These analyses support our conclusions that the regions examined represent regulatory regions that LSD1 decommissions.

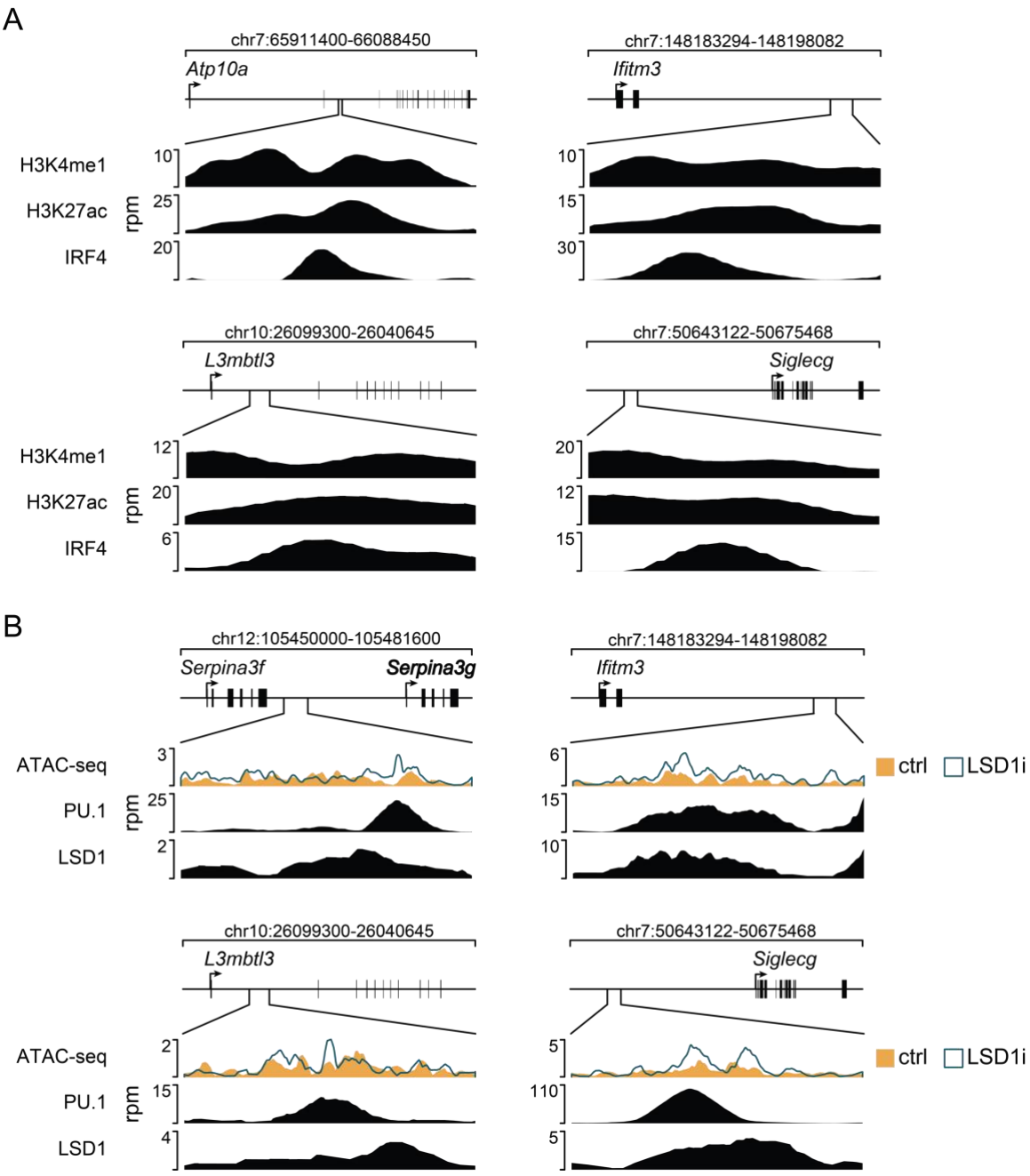


Figure 2-14 – Validation of LSD1-regulated chromatin
(A) Gene tracks of DAR that contain reads per million (rpm) enrichment of H3K4me1, H3K27ac, and IRF4 determined by ChIP-seq in $CD8^+$ T cells[339]. **(B)** Gene tracks of DAR that contain rpm enrichment of chromatin accessibility, PU.1, and LSD1 determined by ATAC-seq and ChIP-seq in mouse-engrafted MLL-AF9 primary acute myeloid leukemia cells[341]. ATAC-seq data are from cells treated (LSD1i) or not treated (ctrl) with an LSD1 inhibitor.

Chapter 3: LSD1 cooperates with NF-κB to regulate marginal zone B cell development

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Abstract

Marginal zone B cells (MZB) are a mature B cell subset that rapidly respond to blood-borne pathogens. Although the transcriptional changes that occur throughout MZB development are known, the corresponding epigenetic changes and epigenetic modifying proteins that facilitate these changes are poorly understood. The histone demethylase LSD1 is an epigenetic modifier that promotes plasmablast formation, but its role in B cell development has not been explored. Here, a role for LSD1 in the development of B cell subsets was explored. B cell-conditional deletion of LSD1 in mice resulted in a decrease in MZB while follicular B cells (FoB) and bone marrow B cell populations were minimally affected. LSD1 repressed genes in MZB that were normally upregulated in the myeloid and FoB lineages. Correspondingly, LSD1 regulated chromatin accessibility at the motifs of transcription factors known to regulate splenic B cell development, including NF-κB motifs. The importance of NF-κB signaling was examined through an *ex vivo* MZB development assay, which showed that both LSD1-deficient and NF-κB-inhibited transitional B cells failed to undergo full MZB development. Gene expression and chromatin accessibility analyses of *in vivo*- and *ex vivo*-generated LSD1-deficient MZB indicated that LSD1 regulated the downstream target genes of non-canonical NF-κB signaling. Additionally, LSD1 was found to interact with the non-canonical NF-κB transcription factor p52. Together, these data reveal that the epigenetic modulation of the non-canonical NF-κB signaling pathway by LSD1 is an essential process during the development of MZB.

Introduction

B cell progenitors develop through multiple stages to become mature naïve B cells capable of generating a humoral immune response. In the bone marrow, common lymphoid progenitors progress through the pro-B and pre-B cell stages, during which the B cell receptor (BCR) is rearranged to generate a functional yet diverse repertoire of B cells[285]. BCR-expressing immature B cells migrate to the spleen where they undergo transitional B cell development, resulting in the formation of follicular B cells (FoB) and marginal zone B cells (MZB). FoB circulate throughout the periphery and facilitate humoral immune responses to antigen and give rise to memory B cells and long-lived plasma cells[285]. MZB localize to the splenic marginal sinus and rapidly respond to blood-borne pathogens, primarily forming short-lived plasmablasts[281,285].

Specific signaling mechanisms drive the MZB or FoB cell fate decision. When immature B cells enter the periphery, they undergo positive selection through tonic BCR signaling to promote survival[342]. The strength of tonic BCR signaling influences immature B cell fate with stronger signals promoting FoB commitment and weaker signals promoting MZB commitment[342]. Immature B cells must experience two additional signaling pathways to further commit to the MZB fate. The first is Notch2 signaling through interaction with the Notch ligand DLL1, which is expressed by splenic venules in the red pulp and marginal zone[285]. The second is BAFFR-dependent activation of non-canonical NF-κB signaling[285]. Both pathways are necessary for MZB cell development and function in a synergistic manner[285].

Throughout cell fate commitment, MZB acquire a transcriptional identity distinct from FoB that confers specific functional capabilities[281,285]. For example, MZB express

high levels of *S1pr1* to facilitate homing to the marginal zone[343] and downregulate the FoB genes *Itgb7*, *Cxcr4*, and *Ccr7* that facilitate homing to secondary lymphoid organs[344]. *Myc* is highly expressed in MZB, providing an enhanced capacity to proliferate in response to antigens such as bacterial lipopolysaccharide (LPS)[345]. MZB can rapidly respond to other TLR agonists[346] and display a concomitant increase in innate immune sensor molecules relative to FoB, including TLR3, TLR7, TLR9, NOD1/2/3, and NLRC4[347]. Although the MZB transcriptome is characterized, the epigenetic modifications acquired during B cell development that establish it are not well studied. Additionally, the enzymes that facilitate splenic B cell epigenetic remodeling are not known.

Lysine-specific demethylase 1 (LSD1) is a histone demethylase that targets H3K4me1, H3K4me2, H3K9me1, and H3K9me2 through FAD-dependent amine oxidation[3]. LSD1-based modification of chromatin results in the fine-tuning of target gene expression, which is critical for driving cellular development[3]. Regarding B cell differentiation, LSD1 promotes plasmablast formation and decommissions active enhancers at Blimp-1, PU-1, and IRF4 binding sites through H3K4me1 demethylation and repression of chromatin accessibility[348]. LSD1 also promotes germinal center formation by repressing plasma cell genes, such as *Prdm1* and *Irf4*, through enhancer decommissioning facilitated by interaction with BCL6[80]. Despite evidence highlighting a critical role for LSD1 in the epigenetic regulation of B cell differentiation, its *in vivo* role during B cell development has not been explored.

In this study, mice with B-cell conditional deletion of LSD1 were used to examine its function throughout B cell development. Phenotyping revealed that LSD1 was dispensable for the development of bone marrow B cell subsets and FoB but was required

for MZB formation. RNA-seq analysis of LSD1-deficient MZB and FoB showed that LSD1 functions as a transcriptional repressor in MZB. Assay for transposase accessible chromatin sequencing (ATAC-seq) analysis revealed a chromatin modulatory role for LSD1 at motifs of transcription factors critical for MZB development, including NF-κB. Experiments using an *ex vivo* MZB development system indicated pathway overlap between LSD1 and non-canonical NF-κB signaling. LSD1 and NF-κB p52 also interact following non-canonical NF-κB stimulation. Overall, these data identify LSD1 as a key transcriptional and epigenetic modifier during MZB development.

LSD1 regulates marginal zone B cell development

LSD1 regulates B cell differentiation to plasma cells[80,348], but its role in B cell development has not been explored. To examine its role from the pro-B to mature B cell stage, CD19-based B cell-conditional LSD1 deletion mice (CKO)[348] and *Cd19*$^{Cre/+}$ control mice (CreWT) were phenotyped by flow cytometry. Compared to CreWT mice, the bone marrow of CKO mice exhibited similar numbers of total B cells, pro-B cells, pre-B cells, immature B cells, and mature B cells (**Fig. 3-1 A-C**). The spleen of CKO mice exhibited similar numbers of total B cells, transitional B cells, and FoB, but there was a 1.5-fold reduction in MZB (**Fig. 3-1 D-F**). A significant reduction in MZB was also observed in CKO mice using two alternative MZB gating strategies (**Fig. 3-1 G-H**). LSD1 protein was not detected in CKO splenic naïve B cells (**Fig. 3-1 I**), confirming knockout in this population. CKO and CreWT spleens were examined by immunofluorescence for markers IgM (total B cells), Cd1d (MZB), and CD169 (marginal zone macrophages) to determine if CKO splenic marginal zones were morphologically normal. Compared to CreWT, CKO

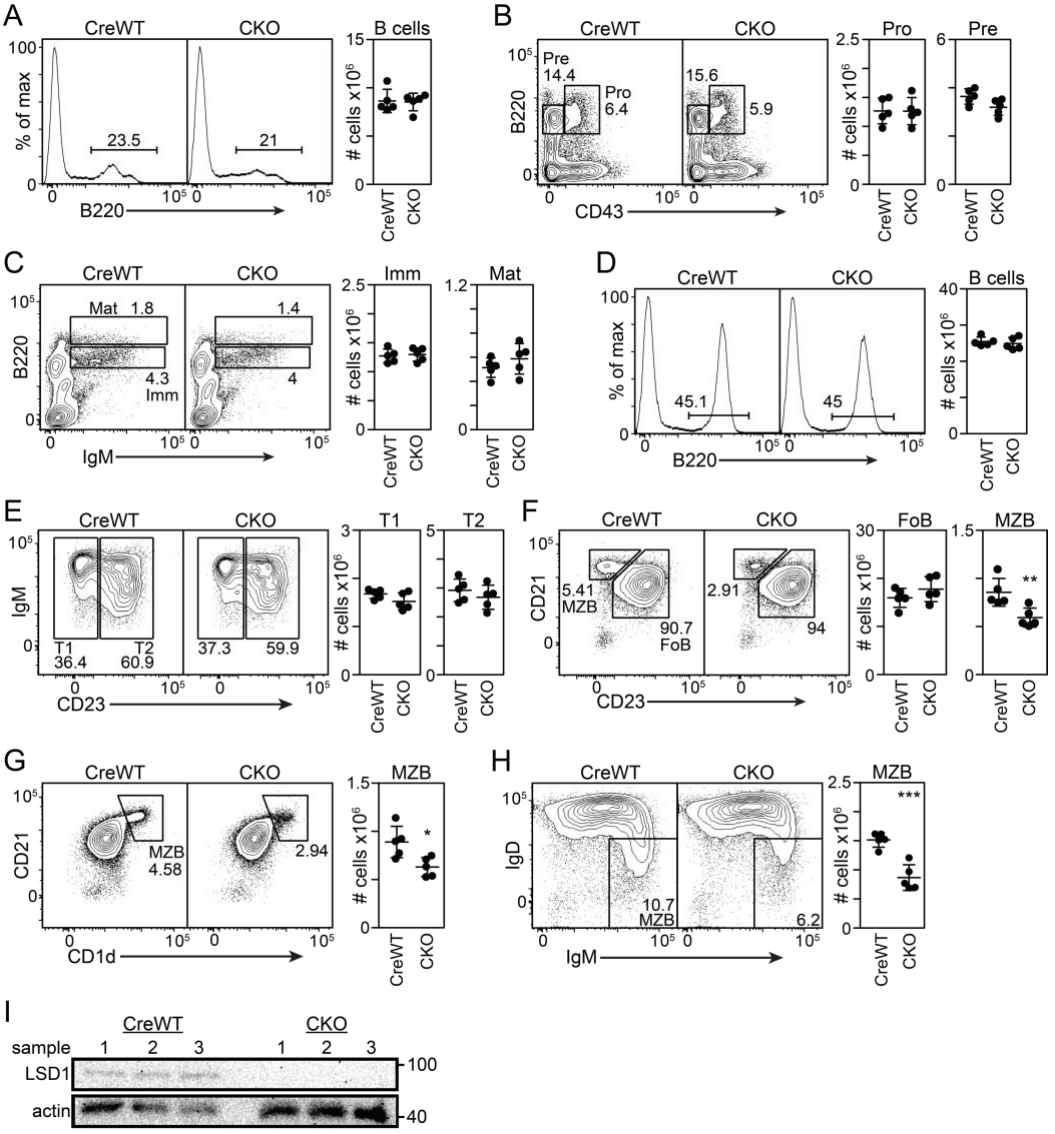


Figure 3-1 – B cell conditional deletion of LSD1 results in fewer marginal zone B cells. **(A-C)** Flow cytometry analysis of the expression of developing B cell markers in the bone marrow of unstimulated naïve CreWT and CKO mice. Analysis of CreWT and CKO total cell numbers of the following B cell populations are shown: **(A)** $B220^+$ B cells; **(B)** $IgM^-B220^+CD43^+$ pro-B cells and $IgM^-B220^+CD43^-$ pre-B cells; **(C)** IgM^+B220^{mid} immature B cells and IgM^+B220^{hi} mature B cells. **(D-H)** Flow cytometry analysis of the expression of developing B cell markers in the spleen of unstimulated CreWT and CKO mice. Analysis of CreWT and CKO total cell numbers of the following B cell populations are shown: **(D)** $B220^+$ B cells; **(E)** $B220^+CD93^+CD23^-$ T1 and $B220^+CD93^+CD23^+$ T2 B cells; **(F)** $B220^+CD93^-CD21^{hi}CD23^-$ MZB and $B220^+CD93^-CD21^{mid}CD23^+$ FoB; **(G)** $B220^+CD93^-CD21^{hi}CD1d^+$ MZB; **(H)** $B220^+CD93^-IgM^+IgD^-$ MZB. **(I)** Western blot of LSD1 protein quantified from splenic naïve B cells purified from three CKO mice and three CreWT mice. All flow cytometry data are representative of at least two independent experiments using three to five mice per group. Error bars represent mean ± SD. Significance was determined by Student's two-tailed *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001.

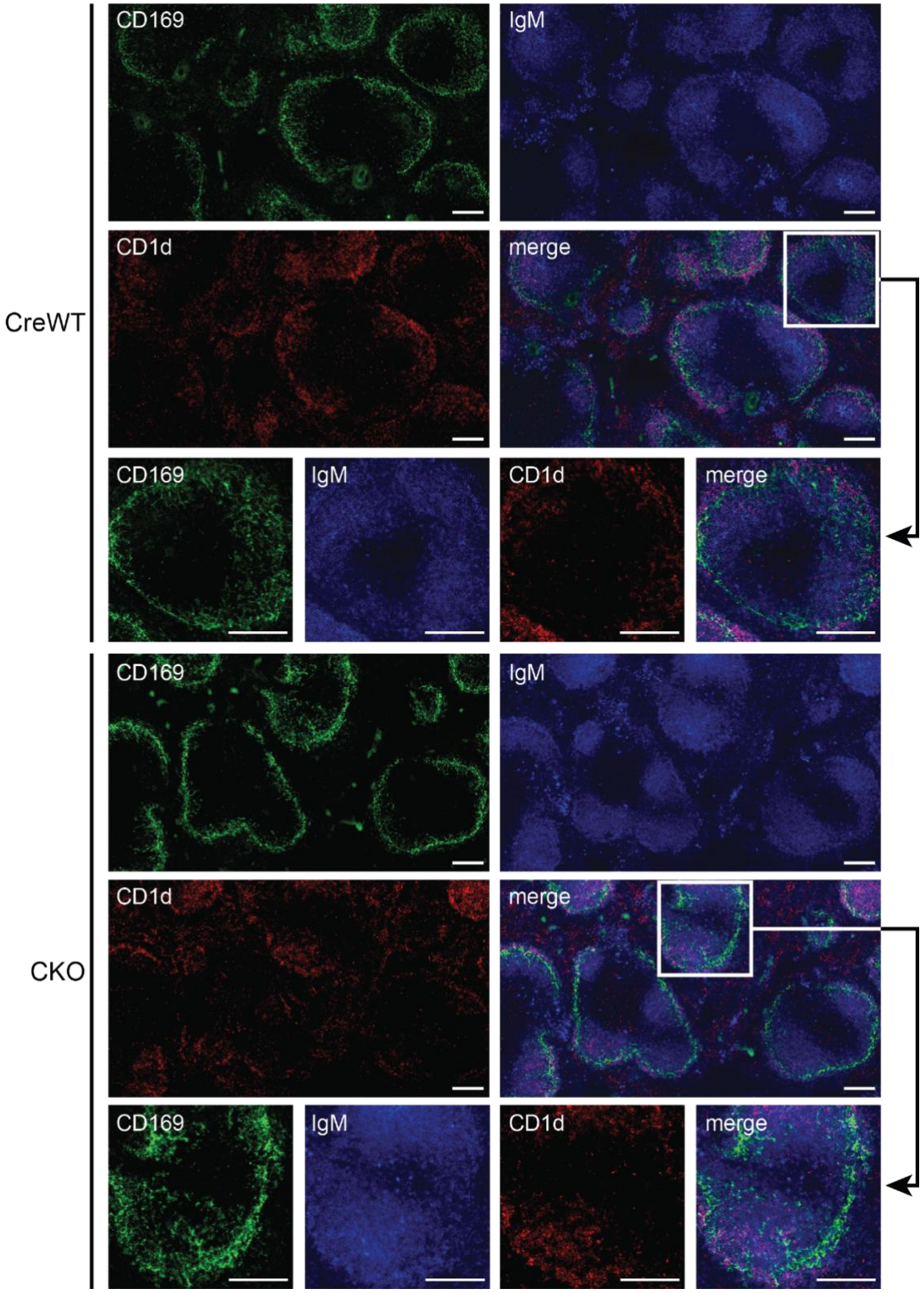

CreWT
CD169
IgM
CD1d
merge
CD169
IgM
CD1d
merge
CKO
CD169
IgM
CD1d
merge
CD169
IgM
CD1d
merge

Figure 3-2 – B cell conditional LSD1 deletion mouse spleens display normal marginal zone architecture

Immunofluorescence staining for CD169 (green), IgM (blue), and CD1d (red) in the spleens of CreWT and CKO mice. Images are at 10X magnification. Scale bars, 400 μm.

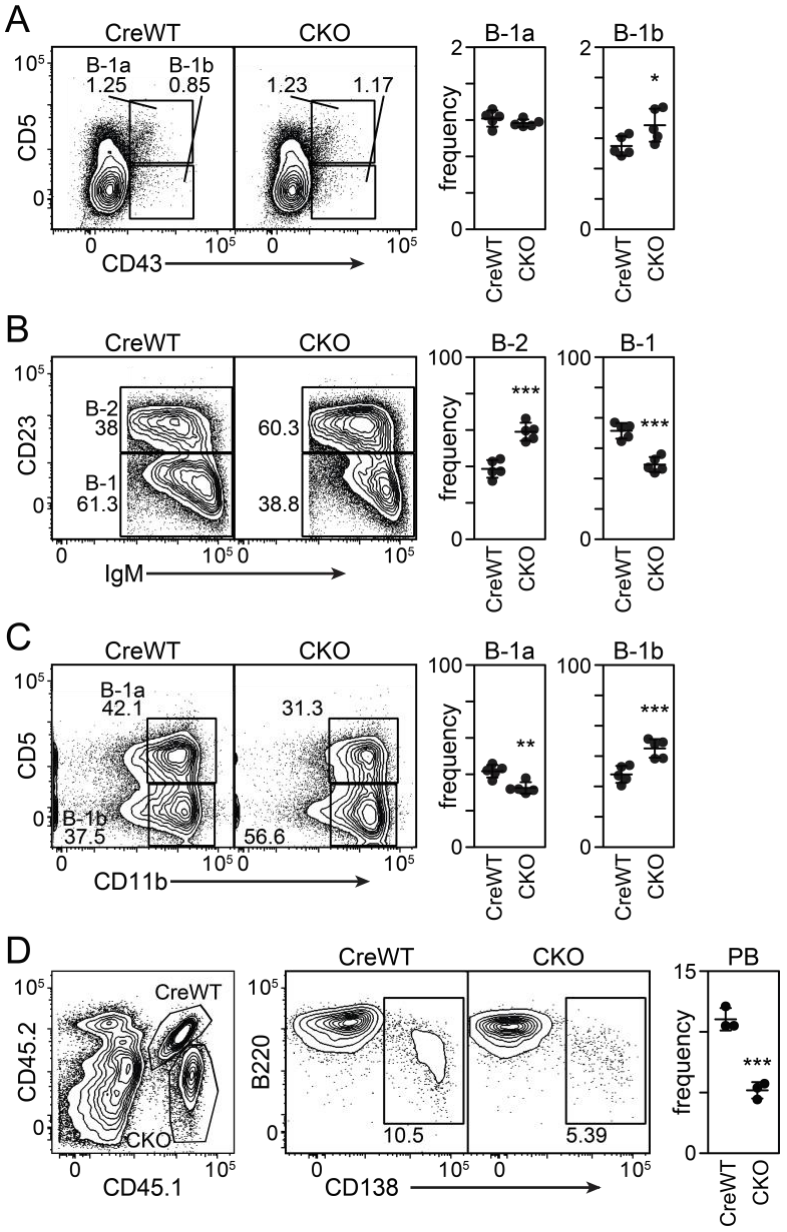


Figure 3-3 – LSD1 regulates B-1 B cell populations.

(A-C) Flow cytometry analysis of the expression of B-1 B cell markers of (A) IgM^+ cells in the spleen, (B) IgM^+ cells in the peritoneal cavity, and (C) IgM^+ $CD23^-$ cells in the peritoneal cavity. (D) Flow cytometry analysis of $CD138^+$ plasmablasts resulting from the LPS-induced differentiation of congenically labeled MZB that were adoptively transferred into μMT host mice. All data are representative of at least two independent experiments

using three to five mice per group. Error bars represent mean ± SD. Significance was determined by Student's two-tailed *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001.

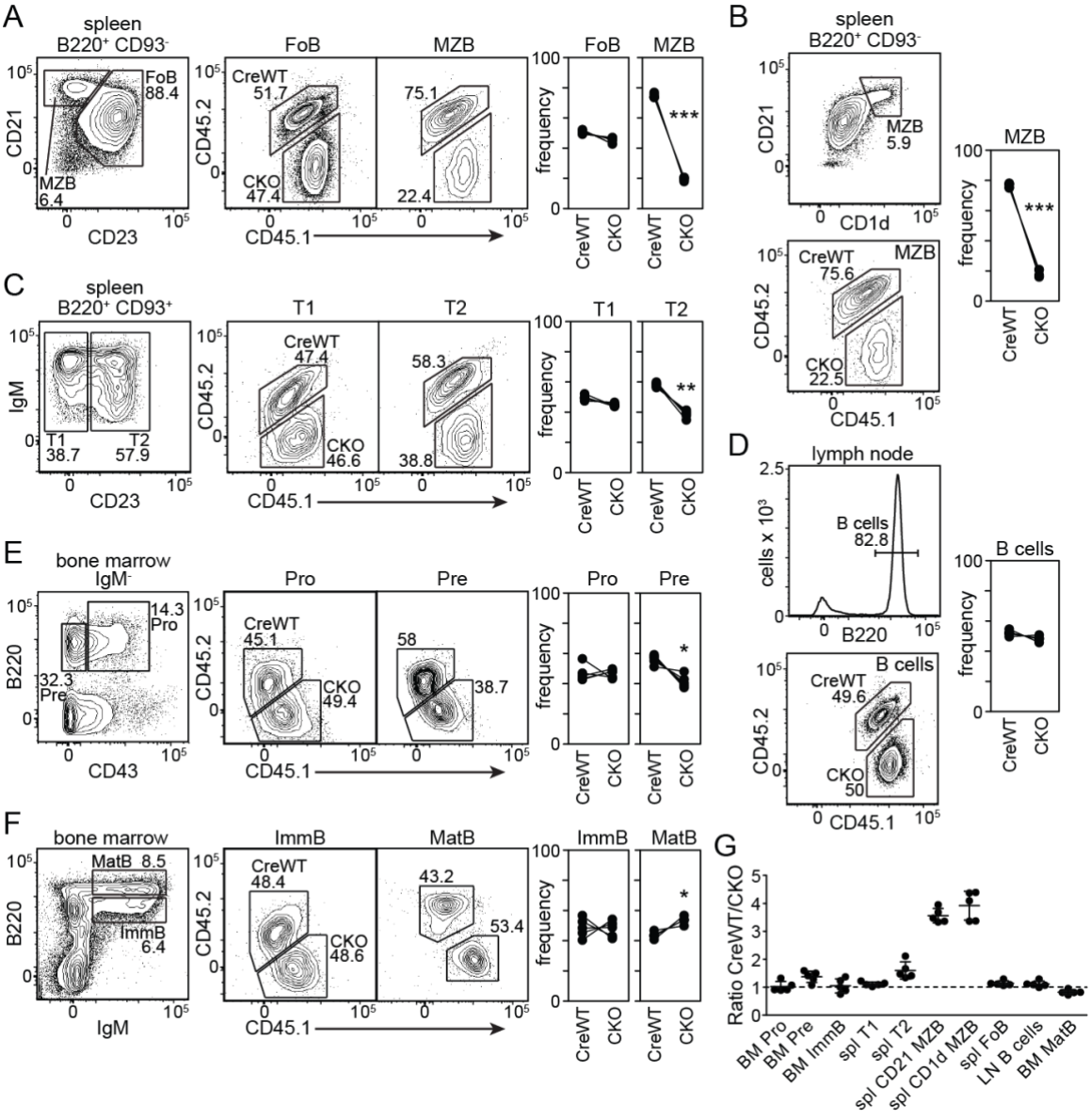


Figure 3-4 – Reduction of marginal zone B cells is cell intrinsic.
(A-F) Flow cytometry analysis of the expression of developing B cell markers in the spleen and bone marrow of unstimulated mixed bone marrow chimera mice reconstituted in a 50:50 ratio of CKO and CreWT bone marrow. Analysis of CreWT and CKO frequencies of the following B cell populations are shown: **(A)** splenic $B220^{+}CD93^{-}CD21^{mid}CD23^{+}$ FoB and $B220^{+}CD93^{-}CD21^{+}CD23^{-}$ MZB; **(B)** splenic $B220^{+}CD93^{-}CD21^{hi}CD1d^{+}$ MZB; **(C)** splenic $B220^{+}CD93^{+}CD23^{-}$ T1 B cells and $B220^{+}CD93^{+}CD23^{+}$ T2 B cells; **(D)** lymph node $B220^{+}$ B cells; **(E)** bone marrow $IgM^{-}B220^{+}CD43^{+}$ pro-B cells and $IgM^{-}B220^{+}CD43^{-}$ pre-B cells; **(F)** bone marrow $IgM^{+}B220^{mid}$ immature B cells and $IgM^{+}B220^{hi}$ mature B cells. **(G)** Ratios of CreWT/CKO frequencies of B cell populations per mouse.

All data are representative of two independent experiments using five mice per group. Error bars represent mean ± SD. Significance was determined by Student's paired two-tailed *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001.

spleens displayed characteristic marginal zone architecture outlined by $CD169^+$ marginal zone macrophages (**Fig. 3-2,** green). White pulp regions also displayed normal patterns of IgM+ B cells and CD1d+ marginal zone B cells (**Fig. 3-2**, blue, red), suggesting that the decrease in CKO MZB is due to a developmental defect instead of a splenic architectural defect. B-1 cell frequencies were assessed in CKO mice (**Fig. 3-3**). Both the spleen (**Fig. 3-3 A**) and the peritoneal cavity (**Fig. 3-3 B,C**) exhibited a significant increase in B-1b cells while the peritoneal cavity exhibited alterations in B-1 and B-2 population frequencies, suggesting that LSD1 regulates B-1 cell development. The ability of LSD1-deficient MZB to respond to the T-independent antigen LPS was examined by adoptively transferring CKO and CreWT MZB in a 1:1 ratio into B cell-deficient μMT host mice. CKO MZB exhibited a two-fold reduction in plasmablast formation (Fig. 3-3 D), verifying the known role that LSD1 has in regulating plasmablast differentiation[348].

To examine the intrinsic nature of CKO B-2 cell development, mixed bone marrow chimeras were established using an equal ratio of CD45.1 CKO and CD45.1/2 CreWT bone marrow in lethally-irradiated CD45.2 wild-type hosts. The frequencies and ratios of reconstituted host B cell compartments were analyzed with flow cytometry. MZB exhibited significantly lower frequencies of CKO cells compared to CreWT (**Fig. 3-4 A,B**), which is reflected in a three- to four-fold reconstitution ratio favoring CreWT cells over CKO cells. Splenic FoB and T1 B cells exhibited similar reconstitution ratios between CreWT and CKO cells, but T2 B cells were skewed in favor of CreWT (**Fig. 3-4 A,C**), supporting a defect in LSD1-deficient splenic B cell development. Lymph node B cells,

pro-B cells, and bone marrow immature B cells also had similar frequencies; however, pre-B cells and bone marrow mature B cells displayed partially skewed reconstitution ratios (**Fig. 3-4 D-F**). Overall, these data demonstrate that LSD1 regulates MZB development in a cell-intrinsic manner (**Fig. 3-4 G**).

LSD1 functions as a transcriptional repressor in marginal zone B cells

The gene regulatory role that LSD1 plays in splenic B cell development was examined by performing RNA-seq on CKO and CreWT MZB and FoB. Principal component analysis (PCA) was performed on all 9,690 detected genes (**Fig. 3-5 A**). Principal component 1 (PC1) stratified CreWT samples by cell type and MZB CreWT from CKO. PC2 further stratified MZB CKO from all samples. Intriguingly, FoB CreWT and CKO samples did not stratify by either PC component, indicating little transcriptional variation due to LSD deletion in those cell types. These data suggest that LSD1 regulates the MZB transcriptional program. Furthermore, the alignment of MZB CKO and FoB cell types suggests that MZB CKO transcriptomes possess FoB-like qualities.

To identify transcriptional differences between cell types and the effects of LSD deletion, differential gene expression analyses were performed on three sample group comparisons: MZB CreWT vs. FoB CreWT, FoB CKO vs. FoB CreWT, and MZB CKO vs. MZB CreWT (**Fig. 3-5 B**). The comparison of MZB and FoB CreWT cells revealed that 1,887 genes that were significantly upregulated in MZB whereas only 101 genes were significantly upregulated in FoB, confirming that MZB and FoB possess distinct transcriptomes[292,347]. Known MZB genes were upregulated in MZB CreWT, including the homing receptor *S1pr1*, the transcription factor *Myc*, and the NOTCH2 target

Dtx1[343,345,349]. Similarly, known FoB genes were upregulated in FoB CreWT, including the homing receptors *Ccr7*, *Cxcr4*, and *Itgb7*, as well as the transcription factors *Bach2* and *Klf2*[344,350,351]. Using GSEA[352], the data above were compared to two previous MZB studies[292,347] (**Fig. 3-5 C**). Genes upregulated in MZB CreWT were significantly enriched for previously identified MZB genes while genes upregulated in FoB CreWT were significantly enriched for previously identified FoB genes, validating the datasets. Comparisons between CKO and CreWT samples for each cell type identified 323 differentially expressed genes (DEG) between MZB CKO and MZB CreWT but only 48 DEG between FoB CKO and FoB CreWT, supporting the conclusion from the PCA that LSD1 primarily regulates the MZB transcriptome. MZB CKO had 297 up DEG and only 26 down DEG, suggesting that LSD1, similar to plasmablasts and germinal center B cells[80,348], mainly plays a repressive role in regulating MZB transcription.

DEG across all samples were assessed and organized based on function (**Fig. 3-5 D**). Signaling genes upregulated in MZB CKO that are known to play a role in B cell development included *Cdkn2c* and *Flt3*. CDKN2C is a cyclin-dependent kinase inhibitor that suppresses cell cycle G1 progression and is known to regulate splenic B cell homeostasis[353]. Loss of FLT3-ligand in mice results in a cell extrinsic increase in MZB and decrease in FoB, suggesting an important role for FLT3 signaling in splenic B cell development[354]. Genes encoding transcriptional regulators important for B cell development were overexpressed in MZB CKO. These included the transcription factors BACH2 and BCL6, which are critical for bone marrow B cell development[350]; ID3, which promotes MZB formation by inhibiting basic helix-loop-helix transcription factors such as E2A[355]; and IRF1 and IRF7, which regulate the expression of interferon response genes[356].

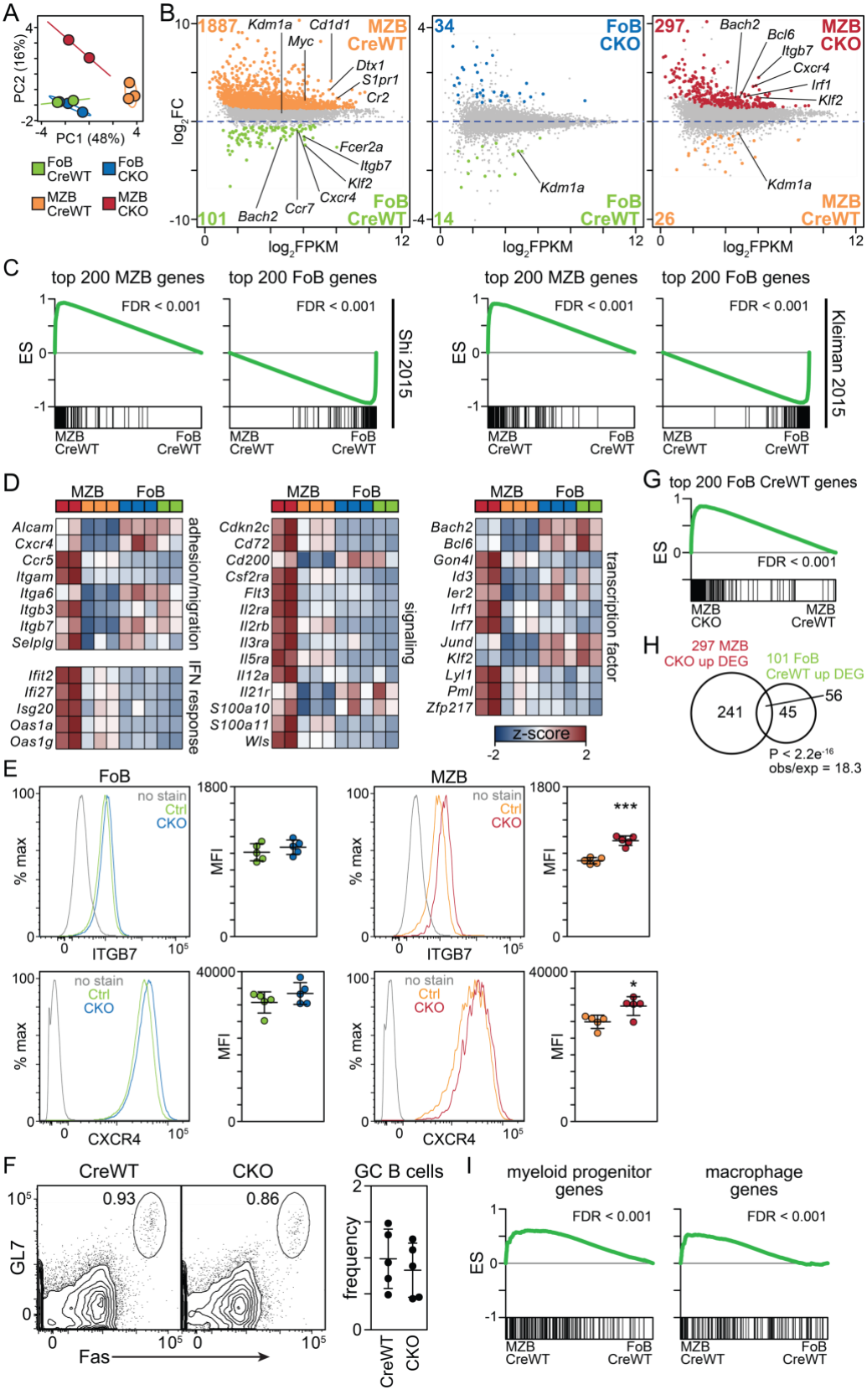

A
PC2 (16%)
PC1 (48%)
FoB CreWT
FoB CKO
MZB CreWT
MZB CKO
B
1887
Kdm1a
Cd1d1
Myc
MZB CreWT
Dtx1
S1pr1
Cr2
Fcer2a
Itgb7
Klf2
Cxcr4
Ccr7
Bach2
101
FoB CreWT
log2FC
log2FPKM
34
FoB CKO
Kdm1a
14
FoB CreWT
297
Bach2
Bcl6
Itgb7
Cxcr4
Irf1
Klf2
MZB CKO
Kdm1a
26
MZB CreWT
C
top 200 MZB genes
top 200 FoB genes
FDR < 0.001
ES
MZB CreWT
FoB CreWT
Shi 2015
Kleiman 2015
D
MZB
FoB
Alcam
Cxcr4
Ccr5
Itgam
Itga6
Itgb3
Itgb7
Selplg
adhesion/migration
Ifit2
Ifi27
Isg20
Oas1a
Oas1g
IFN response
Cdkn2c
Cd72
Cd200
Csf2ra
Flt3
Il2ra
Il2rb
Il3ra
Il5ra
Il12a
Il21r
S100a10
S100a11
Wls
signaling
Bach2
Bcl6
Gon4l
Id3
Ier2
Irf1
Irf7
Jund
Klf2
Lyl1
Pml
Zfp217
transcription factor
z-score
-2
2
G
top 200 FoB CreWT genes
FDR < 0.001
MZB CKO
MZB CreWT
H
297 MZB CKO up DEG
101 FoB CreWT up DEG
241
45
56
P < 2.2e-16
obs/exp = 18.3
E
FoB
MZB
no stain
Ctrl
CKO
% max
MFI
ITGB7
CXCR4
***
*
F
CreWT
CKO
0.93
0.86
GL7
Fas
GC B cells
frequency
I
myeloid progenitor genes
macrophage genes
FDR < 0.001

Figure 3-5 – Reduction of marginal zone B cells is cell intrinsic.
(A) Top two principal components from PCA of z-score normalized mRNA/cell expression of all 9,690 detected genes in all samples. **(B)** Scatterplots of log_2FC vs. log_2FPKM data from differential expression analysis comparing MZB CreWT (orange) and FoB CreWT (green), FoB CKO (blue) vs. FoB CreWT (green), and MZB CKO (red) vs. MZB CreWT (orange). **(C)** Heatmaps of z-score normalized mRNA/cell expression of genes in KEGG pathways or functional categories. **(D)** GSEA plots displaying the enrichment of the top 200 most significant genes upregulated in MZB or FoB from two different studies (Shi 2015[292] and Kleiman 2015[347]) within the MZB CreWT vs. FoB CreWT ranked gene list. **(E)** Flow cytometry analysis of ITGB7 or CXCR4 expression on MZB and FoB that are CKO or CreWT. **(F)** Flow cytometry analysis of germinal center B cell markers GL7 and Fas on B220+ splenic cells that are CKO or CreWT. **(G)** GSEA plot displaying the enrichment of the top 200 most significant genes upregulated in MZB CreWT relative to FoB CreWT within the MZB CKO vs. MZB CreWT ranked gene list. **(H)** Overlapping DEG between the indicated comparison. Significance was determined by Fisher's exact test. Observed/expected (obs/exp) refers to the ratio of observed DEG overlap over expected overlap according to a permutation test. **(I)** GSEA plots displaying the enrichment of myeloid progenitor genes (48) and macrophage genes (49) within the MZB CreWT vs. FoB CreWT ranked gene list. All flow cytometry data are representative of at least two independent experiments using three to five mice per group. Error bars represent mean ± SD. Significance was determined by Student's two-tailed *t*-test. $*P<0.05$, $***P<0.001$.

Genes encoding surface proteins involved in adhesion and migration were upregulated as well and included *Cxcr4* and *Itgb7*, which facilitate homing to the bone marrow and gut, respectively[344]. These two genes exhibited a significant increase in surface expression on MZB (**Fig. 3-5 E**), validating the gene expression data. BACH2 and BCL6 are also known to promote germinal center B cell formation[357], but there was no significant increase in germinal center B cells in CKO spleens (**Fig. 3-5 F**).

Genes critical for FoB function such as *Klf2*, *Bach2*, *Itgb7*, and *Cxcr4* were upregulated in MZB CKO, implying defective MZB development through aberrant expression of FoB genes. To further explore this effect, the top 200 significant genes upregulated in FoB CreWT compared to MZB CreWT were analyzed for enrichment in MZB CKO genes using GSEA (**Fig. 3-5 G**). The results displayed a significant enrichment

of FoB CreWT genes in the MZB CKO cells. Furthermore, the 297 up DEG in MZB CKO were tested for significant overlap with the 101 up DEG in FoB CreWT (**Fig. 3-5 H**). A total of 56 genes overlapped between the two groups, which was 18.3-fold more than expected by chance. Additional example FoB genes include those that encode JUND, a transcription factor that promotes *Bcl6* expression in germinal center B cells[358], CD200, a receptor that is overexpressed on B cell neoplasms and regulates anti-tumor immunity[359], and IL21R, which binds IL-21 to regulate B cell proliferation, differentiation, and survival[360]. Also, because some MZB CKO upregulated DEG are highly expressed in myeloid cells, such as *Csf2ra*, *Irf1*, *Irf7*, and *Itgam*, and pre-B cells can be transdifferentiated into macrophages by altering the pre-B cell transcriptional network[361], MZB CKO genes were tested for enrichment of myeloid progenitor[362] and macrophage[363] lineage genes (**Fig. 3-5 I**). MZB CKO were significantly enriched for these gene sets, suggesting that in the absence of LSD1, there is lineage dysregulation into a myeloid-type cell. Thus, LSD1 is important for establishing the transcriptional identity of MZB during splenic B cell development, partly through repressing FoB and myeloid lineage transcriptional programs.

LSD1 represses chromatin accessibility at NF-κB motifs

The effect that LSD1 deficiency has on chromatin accessibility in FoB and MZB was addressed by performing ATAC-seq on the same sample groups as RNA-seq. PCA on all 94,161 peaks showed that samples separated by both cell type and LSD1 deletion status (**Fig. 3-6 A**). MZB CKO samples separated more from MZB CreWT samples by

PC2 compared with the separation between FoB CKO and CreWT samples, suggesting that LSD1 has a larger impact on chromatin accessibility in MZB than FoB.

Differential accessibility analysis was performed on three sample group comparisons: MZB CreWT vs. FoB CreWT, FoB CKO vs. FoB CreWT, and MZB CKO vs. MZB CreWT (**Fig. 3-6 B**). Comparison of FoB CreWT and MZB CreWT revealed thousands of differentially accessible regions (DAR), indicating that these cell types are very distinct at the level of chromatin accessibility. Compared to their CreWT counterparts, MZB CKO had 1,014 total DAR while FoB CKO had 678 total DAR, with DAR increasing in accessibility being more numerous than DAR decreasing in accessibility for both comparisons. Thus, LSD1 regulates chromatin accessibility in both MZB and FoB – but to a greater extent in MZB – and plays more of a repressive role in both cell types.

To understand what transcription factor binding motifs were enriched within DAR, motif enrichment analysis was performed and enrichment *P* values for top motifs were plotted as a heatmap for the CreWT, FoB CKO, and MZB CKO sample group comparisons (**Fig. 3-6 C-E**). For the MZB and FoB CreWT comparison (**Fig. 3-6 C**), ETS factor motifs were highly enriched in both DAR groups. ETS factors such as SPIB, SPI1, ETS1, and FLI1 are known to regulate splenic B cell development[364-366] and may be influencing chromatin accessibility in these cell types. Transcription factor binding motifs for bHLH, POU, Rel homology domain (RHD), and RUNT factors were more enriched in MZB CreWT DAR, suggesting a role for these factors in regulating MZB chromatin accessibility. Certain bHLH factors such as TCF3 (E2A), TCF4 (E2-2), and MYC regulate the formation and function of splenic B cells[345,355,367]. Both POU factors and RHD factors

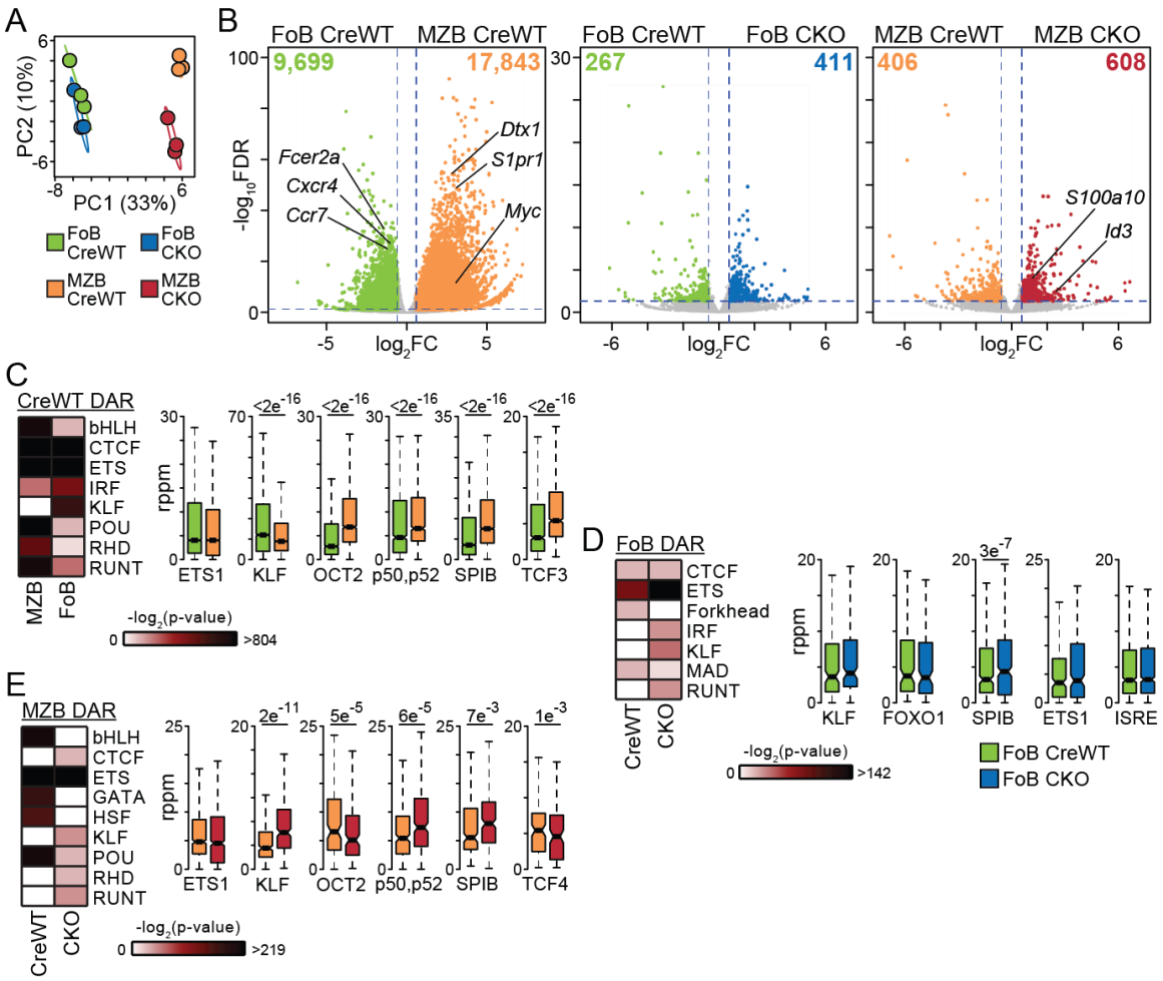


Figure 3-6 – LSD1 regulates chromatin accessibility during splenic B cell development

(A) Top two principal components from PCA of z-score normalized rppm accessibility data of all 94,161 detected peaks in all samples. **(B)** Volcano plots of -$\log_{10}$FDR vs. $\log_2$FC from differential accessibility analysis on three different sample group comparisons. **(C,D,E)** Heatmap displaying –$\log_2$(*P* values) of top significantly enriched transcription factor family motifs for the indicated DAR groups from B. Boxplots depict rppm enrichment of chromatin accessibility for the indicated sample group at specific transcription factor motifs. Significance was determined by Student's two-tailed *t*-test.

are required for normal splenic B cell development[368-370]. IRF and KLF factor motifs were more enriched in FoB CreWT DAR. Since KLF2 and IRF4 activate genes important for follicular B cell function[351,356], they may influence chromatin accessibility to exert their transcriptional regulation.

For the CreWT vs. CKO comparisons (**Fig. 3-6 D,E**), ETS factor motifs were highly enriched in all DAR groups. LSD1 has been shown to modulate chromatin accessibility at ETS motifs and binding sites in plasmablasts or a B cell line[348,371], providing further evidence for B cell-specific LSD1-based regulation at these sites. MZB CKO up DAR were enriched for CTCF, KLF, RHD, and RUNT binding motifs, whereas MZB CreWT DAR were enriched for bHLH, GATA, HSF, and POU binding motifs. LSD1 has been shown to interact directly with the bHLH factors MYOD and TAL1[92,372] and the RHD factor p65[24], supporting the possibility that LSD1 may cooperate with transcription factors of the same families during B cell development to exert its effects on chromatin accessibility and gene expression.

PageRank analysis was used to integrate RNA-seq data and ATAC-seq data to rank transcription factors in each sample group by predicted importance based on the expression and identity of its target genes[373]. The PageRank score of the top 20 transcription factors (out of 639 analyzed) per sample group were plotted as a heatmap (**Fig. 3-7 A**). Reflecting their shared precursor origins, the analysis indicated that FoB and MZB share many top factors, including SPIB, ETS1, ELF1, p50, and p52. Some transcription factors are unique to certain sample groups, such as PBX2 to MZB, indicating a more prominent role in target gene regulation for these factors in these sample groups.

PageRank between MZB CreWT and FoB CreWT (**Fig. 3-7 B**) identified factors known to be important for the formation and function of MZB and/or FoB were identified and include BACH2, BCL6, EBF1, FLI1, MYC, p52, and TCF4[345,350,366,367,369,374]. Other factors identified such as MEIS3 and PBX2 have not been shown to play a role in B cell development, but are known to regulate the development of other cell types[375,376]. Between

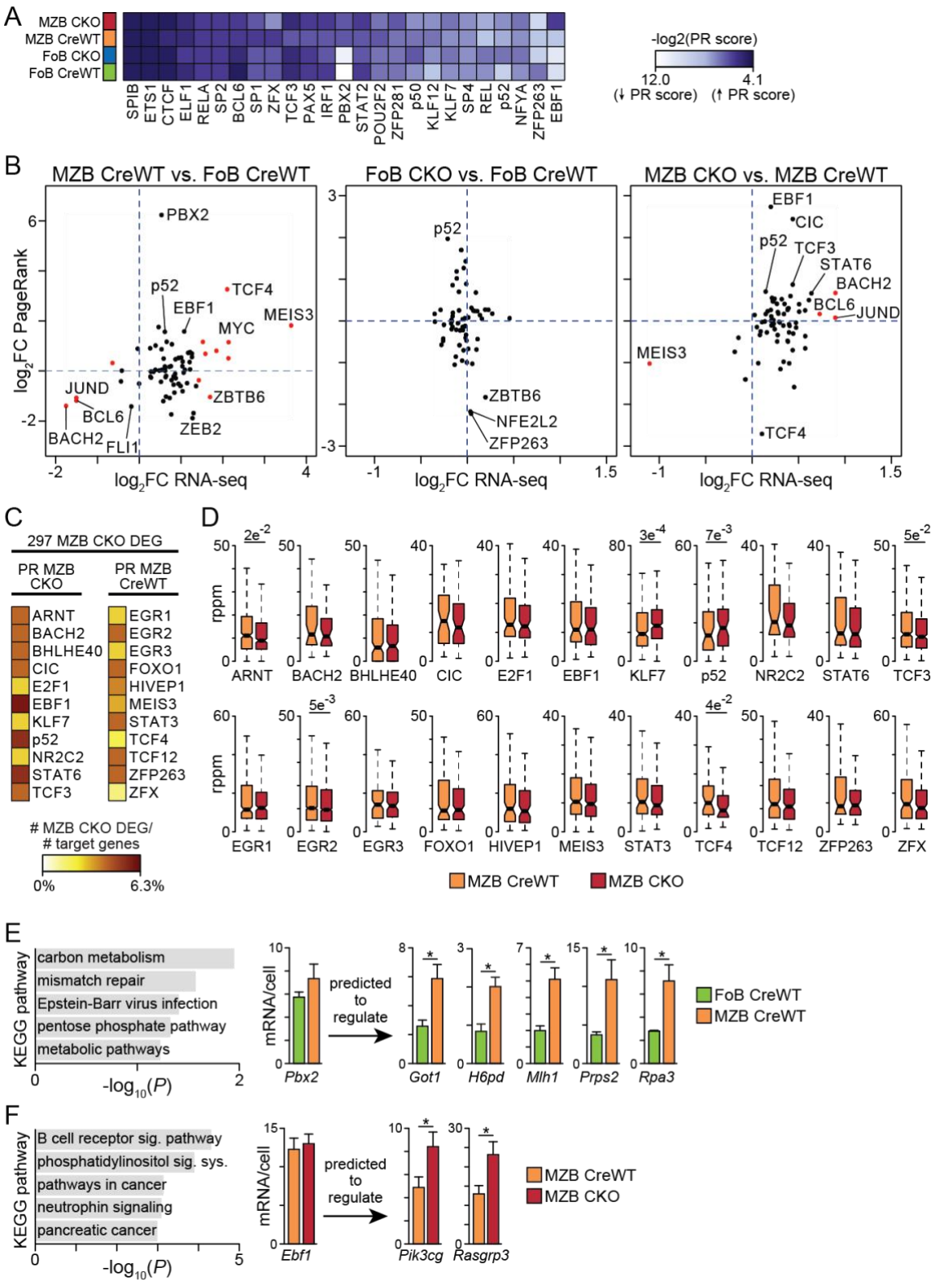


Figure 3-7 – LSD1 regulates chromatin accessibility at transcription factor networks

(A) Heatmap of the $-\log_2$(PageRank) score of the top 20 transcription factors per sample group. **(B)** Scatterplots of $\log_2$FC data from PageRank analysis vs. $\log_2$FC data from RNA-seq analysis on three different sample group comparisons. Points correspond to transcription factors with a PageRank score >0.003 (top 10%) in at least one of the compared sample groups. A red point indicates a DEG in the given comparison. **(C)** Heatmaps displaying the percentage of MZB CKO DEG in total predicted target genes of the transcription factors listed. PR MZB CKO transcription factors have a $\log_2$FC PageRank score > 0.5 while PR MZB CreWT transcription factors have a $\log_2$FC PageRank score of < -0.5. **(D)** Boxplot rppm enrichment of chromatin accessibility at the indicated motifs mapping to DEG that are predicted target genes of the transcription factor that binds to the motif. **(E)** Top five KEGG pathways of PBX2 target genes, expression of *Pbx2*, and example predicted target genes. **(F)** Top five KEGG pathways of EBF1 target genes, expression of *Ebf1*, and example predicted target genes.

FoB CKO and FoB CreWT, transcription factors such as NF-κB p52 were determined to be more important in FoB CKO despite the relatively unchanged FoB CKO transcriptome and lack of a splenic FoB phenotype in CKO mice. Between MZB CKO and MZB CreWT, the transcription factors BACH2, CIC, EBF1, p52, STAT6, and TCF3 were determined to be more important to the MZB CKO transcriptional program, implying LSD1-dependent regulation of their downstream target genes.

The transcription factors most likely to cooperate with LSD1 to directly regulate target genes through modulation of chromatin accessibility were determined by filtering MZB CKO by PageRank score (> 0.5 or < –0.5), and then analyzing filtered transcription factors for 1) highest percent DEG of all target regulated genes, and 2) chromatin accessibility changes at motifs mapping to these DEG. EBF1, p52, and STAT6 had the highest percent of MZB CKO DEG of their target regulated genes (**Fig. 3-7 C**). Of these factors, only p52 exhibited a significant increase in accessibility in MZB CKO compared to MZB CreWT (**Fig. 3-7 D**), suggesting that without LSD1, p52 fails to properly repress target gene expression and chromatin accessibility, possibly through a direct interaction.

The biological roles of transcription factors were explored by examining their PageRank-determined target genes. PBX2, which was identified as important in MZB CreWT compared to FoB CreWT, is not known to have a role in B cell development. Here, PBX2 was predicted to upregulate 59 DEG in MZB CreWT that were determined to be involved in a number of processes via KEGG pathway analysis, the most significant being carbon metabolism and mismatch repair (**Fig. 3-7 E**). Example genes include the metabolic enzymes *Got1*, *H6pd*, and *Prps2* and the DNA repair enzymes *Mlh1* and *Rpa3*. These data suggest a novel role for PBX2 in marginal zone B cell function. In MZB CKO, EBF1 was predicted to have a dysregulated transcriptional network upon LSD1 deletion despite not having dysregulated chromatin accessibility at target motifs, suggesting an indirect LSD1-mediated regulatory effect. EBF1 is known to regulate genes involved in the B cell receptor signaling pathway and does so in an LSD1-dependent manner with the genes *Pik3cg* and *Rasgrp3* (**Fig. 3-7 F**), suggesting a possible role for LSD1 in regulating this process[374]. p52 was predicted to upregulate 23 DEG in MZB CKO, including the transcriptional regulators BACH2 and ID3, the receptors S100a10 and TLR2, and the signaling molecules PIK3CG, RASGRP3, and SPATA13 (**Fig. 3-8 A**). p52 binding motifs were assessed individually for overlap with DAR. Four of these were identified in the p52-target DEGs *Crisp3*, *Id3*, *S100a10*, *and Sapcd1* (**Fig. 3-8 B**). Nineteen other p52 motif-containing DAR were located throughout the genome (**Fig. 3-8 C**). In addition to the above, 10 DAR that did not contain a p52 motif mapped to p52-target DEG (*Setbp1* and *Tlr2*) (**Fig. 3-8 D**). Together, these data suggest a role for LSD1 in directly repressing the expression of p52-target genes by limiting chromatin accessibility at p52 binding sites.

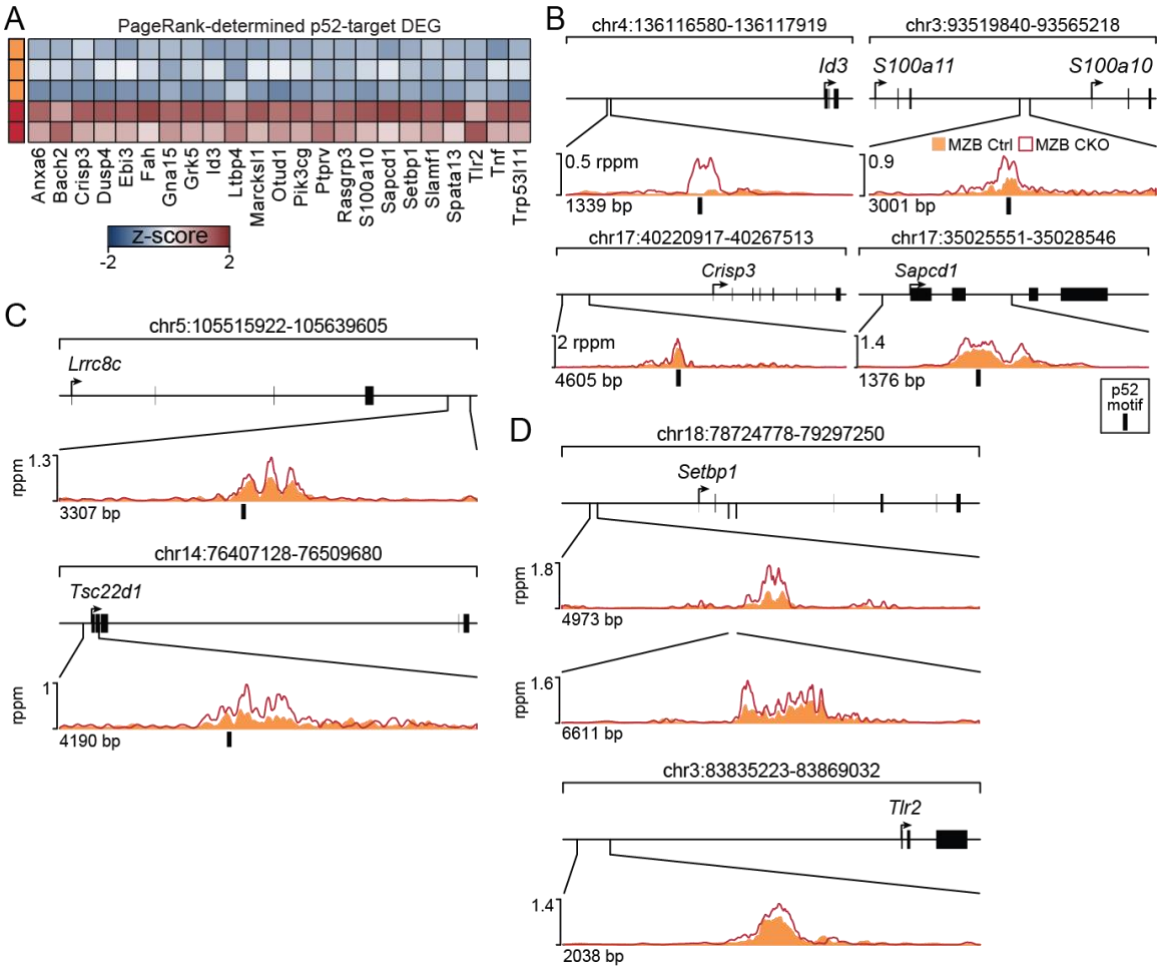


Figure 3-8 – LSD1 regulates chromatin accessibility at p52 motifs.
(A) Heatmap of z-score normalized mRNA/cell expression of p52-target genes predicted by PageRank analysis. **(B,C,D)** Gene track examples of rppm chromatin accessibility data for **(B)** DAR that increase in accessibility in MZB CKO that map to a p52 motif and a p52-target DEG predicted by PageRank analysis, **(C)** DAR that increase in accessibility in MZB CKO that map to a p52 motif, and **(I)** DAR that map to a p52-target MZB CKO upregulated DEG.

LSD1 regulates *ex vivo* marginal zone B cell development induced by NOTCH2 and non-canonical NF-κB signaling

The above analysis suggested a dependence of LSD1 on non-canonical NF-κB signaling through p52, a critical factor for MZB formation[368]. To assess the relevance of LSD1 in non-canonical NF-κB signaling during MZB development CKO and CreWT

$B220^+CD93^+$ transitional stage B cells (TrB) were cultured on OP9-DL1 cells, which stimulate NOTCH2 signaling through delta-1 ligand expression; and in the presence of BAFF to stimulate non-canonical NF-κB signaling[285]. Pre-cultured TrB displayed similar population levels between CKO and CreWT mice and exhibited low surface expression of the MZB surface markers CD21 and CD1d (**Fig. 3-9 A, B**). After 3 days in culture, 14-20% of all CreWT TrB developed into $B220^+CD21^+CD1d^+$ *ex vivo*-derived MZB (eMZB), whereas only 6-10% of CKO TrB developed into eMZB (**Fig. 3-9 C**). Cells were developed under additional conditions, including controls for BAFF and the delta-1 ligand DL1 (**Fig. 3-9 D**). LSD1-deficient cells developed into significantly fewer MZB under all conditions, suggesting a defect in both NOTCH2 and non-canonical NF-κB signaling. To ensure that the defect was due to the absence of LSD1 in splenic B cell development and not earlier stages, $Kdm1a^{fl/fl}Rosa26^{CreERT2/+}$ (IKO) and $Kdm1a^{fl/fl}Rosa26^{+/+}$ (WT) $CD93^+$ TrB were cultured *ex vivo* as above at day five after tamoxifen treatment (**Fig. 3-9 E, F**). The same defect was observed, indicating that the reduction in eMZB CKO cells is likely due to a defect in splenic B cell development.

To determine whether LSD1 regulated NOTCH2-target and/or NF-κB-target genes during eMZB development, RNA-seq was performed on LSD1-deficient and -sufficient eMZB and TrB. PCA indicated that TrB CreWT stratified from eMZB CreWT but not eMZB CKO (**Fig. 3-10 A**), suggesting that changes induced by NOTCH2 and/or NF-κB signaling normally observed in eMZB CreWT are not occurring in eMZB CKO. Minimal stratification was observed between TrB CKO and TrB CreWT, indicating that LSD1 does not have a strong role in regulating the TrB transcriptional program. Differential expression analysis was performed on the indicated sample groups (**Fig. 3-10 B**). The

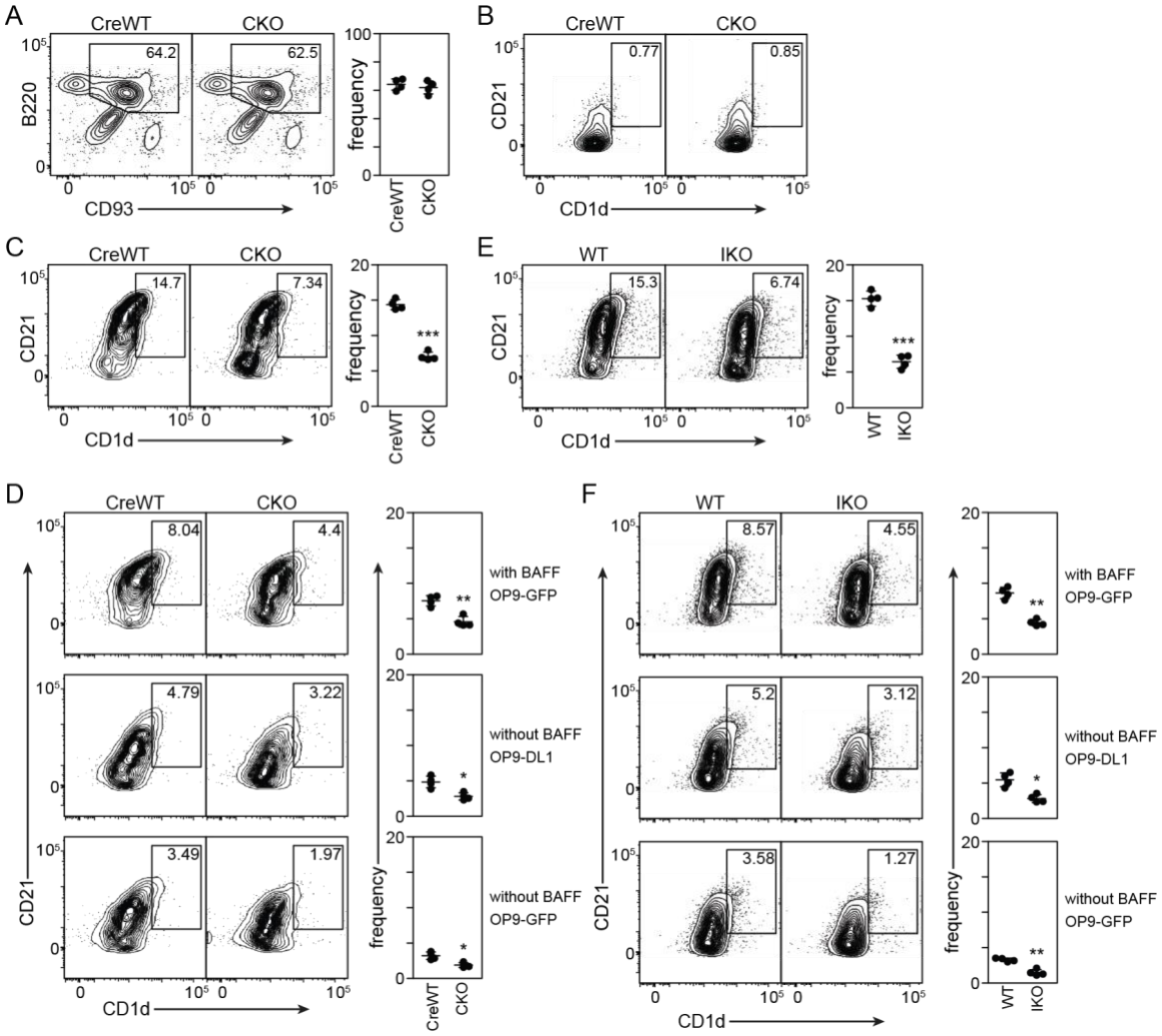


Figure 3-9 – LSD1 regulates *ex vivo* marginal zone B cell development.
(A) Flow cytometry analysis of B220 and CD93 expression on PE^+ enrichments of CD93-PE-stained spleens from CreWT and CKO mice. **(B)** CD21 and CD1d expression on gated populations from part A. **(C)** CD21 and CD1d expression on $B220^+$ CKO or CreWT cells after three days of being cultured with OP9-DL1 cells in the presence of BAFF. **(D)** Flow cytometry analysis of CD21 and CD1d expression on $B220^+$ CKO or CreWT cells after three days of being cultured in the indicated conditions. **(E)** CD21 and CD1d expression on $B220^+$ IKO or WT cells after three days of being cultured with OP9-DL1 cells in the presence of BAFF. Cells were cultured five days after a five day tamoxifen treatment regimen. **(F)** CD21 and CD1d expression on $B220^+$ IKO or WT cells after three days of being cultured in the indicated conditions. Cells were cultured five days after a five-day tamoxifen treatment regimen.

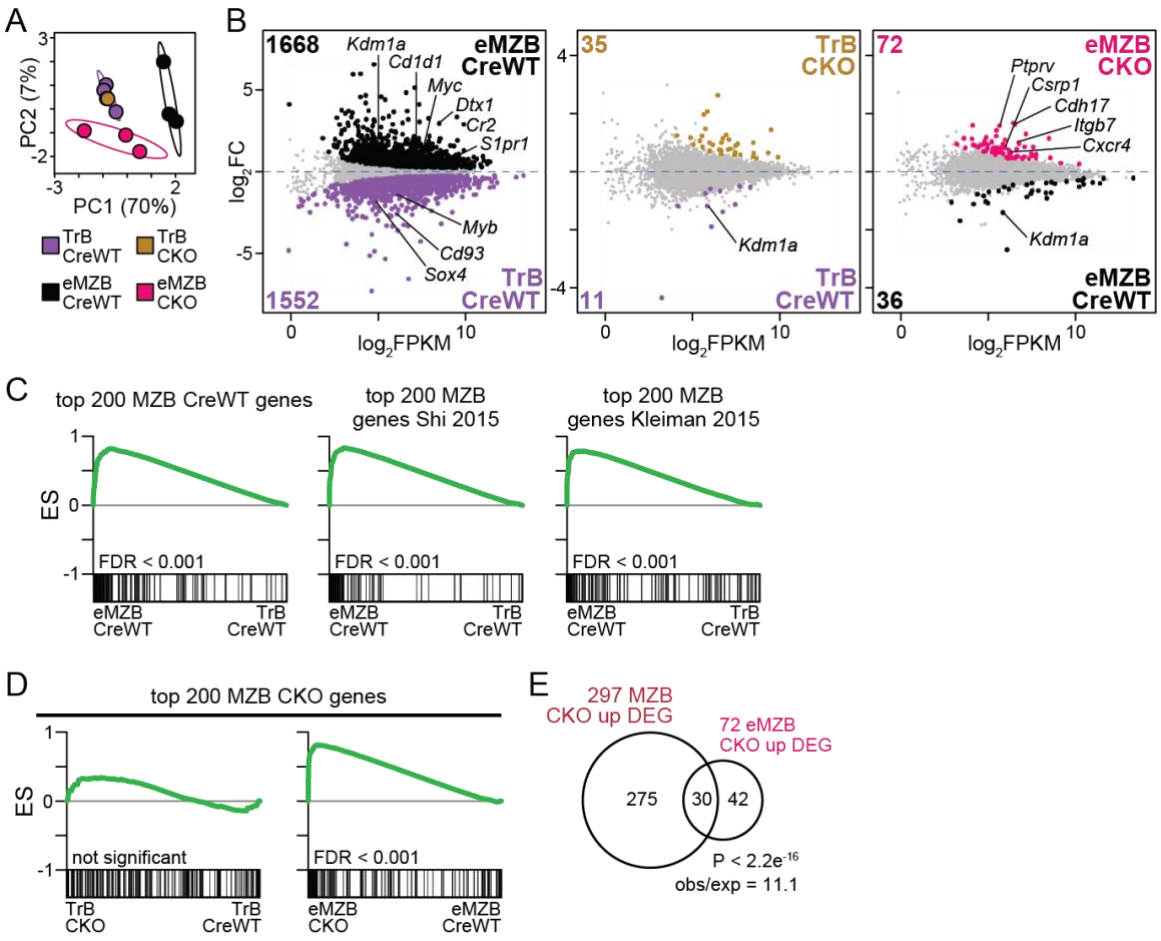
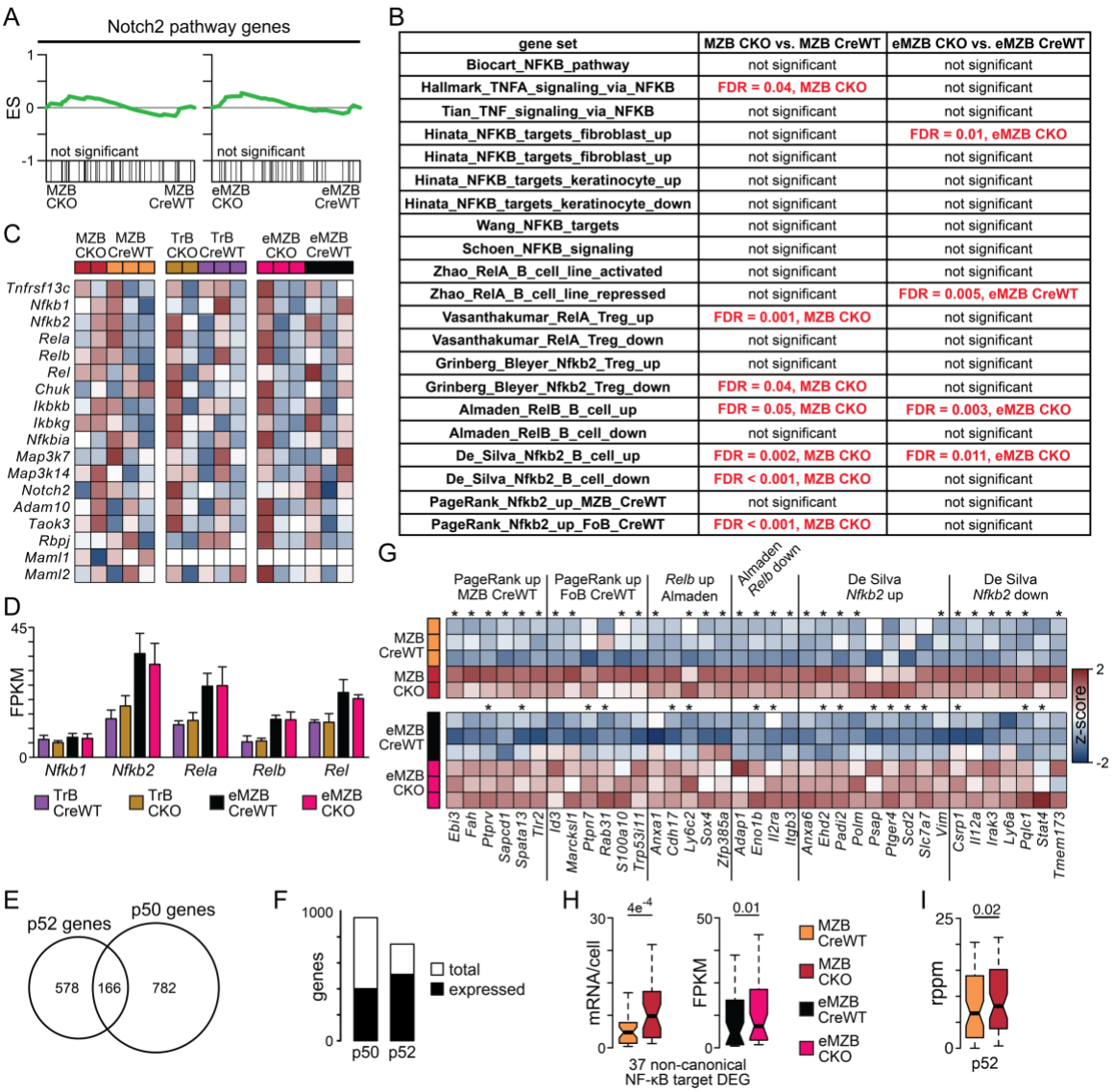


Figure 3-10 – LSD1 regulates the *ex vivo* marginal zone B cell transcriptional program.
(A) Top two principal components from PCA of z-score normalized mRNA/cell expression of all 9,843 detected genes in all samples. **(B)** Scatterplots of log_2FC vs. log_2FPKM data from differential expression analysis for the indicated sample group comparisons. **(C)** GSEA plots displaying the enrichment of the top 200 most significant genes upregulated in MZB from this study and two different studies within the eMZB CKO vs. eMZB CreWT ranked gene list. **(D)** GSEA plots displaying the enrichment of the top 200 most significant genes upregulated in MZB CKO relative to MZB CreWT within two ranked gene lists: TrB CKO vs. TrB CreWT and eMZB CKO vs. eMZB CreWT. **(E)** Overlapping DEG between the indicated comparison. Significance was determined by Fisher's exact test. Observed/expected (obs/exp) refers to the ratio of observed DEG overlap over expected overlap according to a permutation test.

3,220 total DEG observed between TrB CreWT and eMZB CreWT showed that the two cell types were transcriptionally distinct, with eMZB CreWT upregulating MZB genes

such as *Myc*, *Dtx1*, and *S1pr1* and TrB CreWT upregulating transitional B cell genes such as *Myb* and *Sox4*. Using GSEA, MZB genes from this study and two others[292,347] were shown to be significantly enriched in eMZB CreWT genes (**Fig. 3-10 C**), validating the *ex vivo* MZB development assay as a viable method for testing MZB development. CKO comparisons showed 46 total changes in gene expression between TrB CKO and TrB CreWT and 108 total changes in gene expression between eMZB CKO and eMZB CreWT, with most changes being increases (**Fig. 3-10 B)**. eMZB CKO genes, but not TrB CKO genes, were significantly enriched for MZB genes (**Fig. 3-10 D**). Additionally, 30 out of the 72 eMZB CKO upregulated DEG overlapped the 297 MZB CKO upregulated DEG (**Fig. 3-10 E)**, 11.1-fold more than expected by chance), including homing receptors *Cxcr4* and *Itgb7*. These data further support a repressive role for LSD1 during MZB development and suggest a similar role for LSD1 during both *in vivo* and *ex vivo* MZB development. [93]

MZB CKO and eMZB CKO genes were tested for enrichment in NOTCH2 and NF-κB target genes, which were acquired from the Molecular Signatures Database (MSigDB)[377] and, in the case of NF-κB target genes, the PageRank analysis from this study and publications involving genetic deletion of NF-κB signaling transcription factors[368,369,378-380]. NOTCH2 target genes were not significantly enriched in MZB CKO or eMZB CKO (**Fig 3-11 A**). Of all 21 NF-κB target gene sets tested, seven gene sets were significantly enriched in MZB CKO while four gene sets were significantly enriched in eMZB CKO (**Fig. 3-11 B**). Of these sets, two were enriched in both MZB CKO and eMZB CKO and represent genes aberrantly upregulated when either *Nfkb2* or *Relb* are deleted in splenic naïve B cells treated with BAFF. Importantly, genes upstream of both NOTCH2 signaling and NF-κB signaling, such as *Notch2* and the BAFF receptor (*Tnfrsf13c*), were



gene set	MZB CKO vs. MZB CreWT	eMZB CKO vs. eMZB CreWT
Biocart_NFKB_pathway	not significant	not significant
Hallmark_TNFA_signaling_via_NFKB	FDR = 0.04, MZB CKO	not significant
Tian_TNF_signaling_via_NFKB	not significant	not significant
Hinata_NFKB_targets_fibroblast_up	not significant	FDR = 0.01, eMZB CKO
Hinata_NFKB_targets_fibroblast_up	not significant	not significant
Hinata_NFKB_targets_keratinocyte_up	not significant	not significant
Hinata_NFKB_targets_keratinocyte_down	not significant	not significant
Wang_NFKB_targets	not significant	not significant
Schoen_NFKB_signaling	not significant	not significant
Zhao_RelA_B_cell_line_activated	not significant	not significant
Zhao_RelA_B_cell_line_repressed	not significant	FDR = 0.005, eMZB CreWT
Vasanthakumar_RelA_Treg_up	FDR = 0.001, MZB CKO	not significant
Vasanthakumar_RelA_Treg_down	not significant	not significant
Grinberg_Bleyer_Nfkb2_Treg_up	not significant	not significant
Grinberg_Bleyer_Nfkb2_Treg_down	FDR = 0.04, MZB CKO	not significant
Almaden_RelB_B_cell_up	FDR = 0.05, MZB CKO	FDR = 0.003, eMZB CKO
Almaden_RelB_B_cell_down	not significant	not significant
De_Silva_Nfkb2_B_cell_up	FDR = 0.002, MZB CKO	FDR = 0.011, eMZB CKO
De_Silva_Nfkb2_B_cell_down	FDR < 0.001, MZB CKO	not significant
PageRank_Nfkb2_up_MZB_CreWT	not significant	not significant
PageRank_Nfkb2_up_FoB_CreWT	FDR < 0.001, MZB CKO	not significant

Figure 3-11 – LSD1 represses NF-κB target genes.
(A) GSEA plots displaying the enrichment of Notch2 pathway genes within two ranked gene lists: MZB CKO vs. MZB CreWT and eMZB CKO vs. eMZB CreWT. **(B)** GSEA results for the 21 indicated NF-κB gene sets within the two ranked gene lists from A. Red indicates a significantly enriched gene set. **(C)** Heatmaps displaying the expression of components of canonical NF-kB, non-canonical NF-kB, and NOTCH2 signaling pathways. **(D)** FPKM expression of NF-κB transcription factors. **(E)** Venn diagram displaying genes that are regulated by p50 and p52. **(F)** p50 and p52 regulated genes that are expressed in eMZB CreWT samples. **(G)** Heatmaps of z-score normalized mRNA/cell and FPKM expression of 37 genes that are DEG in both sample groups or are a DEG in one group and trending up in the other. DEG are denoted by *. **(H)** Box plots of mRNA/cell or FPKM expression of the 37 genes displayed in D. **(I)** Box plot of rppm enrichment of chromatin accessibility at p52 motifs that map to the 37 genes displayed in D.

not dysregulated in MZB CKO, TrB CKO, or eMZB CKO (**Fig. 3-11 C**). Thus, in the absence of LSD1, these data suggest a defect in non-canonical NF-κB signaling, but not NOTCH2 signaling.

LSD1 and NF-κB cooperate to regulate marginal zone B cell development

Genes regulated by both LSD1 and NF-κB signaling were further analyzed to understand how LSD1 and NF-κB intersect. Of the genes encoding transcription factors that form a functional NF-κB complex, *Nfkb2*, *Rela*, *Relb*, and *Rel* are induced in eMZB compared to TrB while *Nfkb1* is not (**Fig. 3-11 D**), implying that p50 complexes play a lesser role in *ex vivo* MZB development than p52 complexes. This is further supported by the finding that of all p50 and p52 target genes from PageRank analysis and GSEA gene sets used above (**Fig. 3-11 E**), eMZB express 70% of all p52 genes while they only express 42% of all p50 genes (**Fig. 3-11 F**). Genes regulated by p52 complexes in splenic naïve B cells treated with BAFF[368,369] or predicted to be regulated by p52 by PageRank analysis were examined for gene expression changes in MZB CKO and eMZB CKO. A total of 37 genes that were DEG in both sample groups or were a DEG in one group and trending up in the other group are displayed (**Fig. 3-11 G**). Genes include those that encode PTPRV, a protein tyrosine phosphatase that mediates p53-induced cell cycle exit[381], CSRP1, a LIM-domain protein that suppresses cell proliferation and development[382], Ly6A, a surface protein that promotes hematopoietic stem cell development and survival[383], CDH17, a cadherin that regulates early B cell development[384], and ID3, a transcription factor that promotes MZB formation[355]. Total expression of these 37 genes in both MZB CKO and eMZB CKO were significantly higher than their CreWT counterparts (**Fig. 3-11 H**).

Additionally, p52 motifs mapping to these genes exhibit a significant increase in chromatin accessibility in MZB CKO, supporting that they are repressed by LSD1 (**Fig. 3-11 I**).

Non-canonical NF-κB signaling through the transcription factors p52 and RelB is critical for splenic B cell development, as indicated by B cell-conditional knockout of *Nfkb2* and *Relb*[369]. To confirm the role of non-canonical NF-κB signaling during *ex vivo* MZB development, the NF-κB inhibitor IKK-16[385] was applied to *ex vivo* MZB cultures of C57BL/6 wild-type cells (**Fig. 3-12 A**). Cultures treated with 800 nM of IKK-16 exhibited a significant decrease in eMZB compared to control cells treated with DMSO, showing that non-canonical NF-κB signaling is critical for *ex vivo* MZB development and suggesting that both inhibition of NF-κB signaling and LSD1 deficiency affect a similar pathway. To assess pathway overlap, LSD1-deficient cultures were treated with IKK-16 (**Fig. 3-12 B**). CKO inhibitor cultures exhibited a significant decrease in eMZB compared to both CKO DMSO cultures and CreWT inhibitor cultures, but this decrease was not completely additive. These data imply a degree of overlap between pathways affected by both LSD1 deletion and NF-κB inhibition.

To confirm that genes from Fig. 7B are regulated by both LSD1 and NF-κB signaling, RNA was collected from B cells from the four culture conditions displayed in Fig. 7E (**Fig. 3-12 C**) and RT-qPCR was performed to assess the expression of the nine genes that are DEG in both MZB and eMZB (*Csrp1*, *Ehd2*, *Eno1b*, *Il2ra*, *Ly6c2*, *Padi2*, *Pqlc1*, *Ptprv*, *Spata13*). The genes *Ccr7*, *JunB*, and *Tap1*, which are known targets of canonical NF-κB signaling[386-388], were used as negative controls. RT-qPCR revealed that the genes *Csrp1*, *Il2ra*, *Ly6c2*, *Padi2*, *Ptprv*, and *Spata13* were significantly upregulated in CreWT inhibitor cultures compared to CreWT DMSO cultures, indicating their

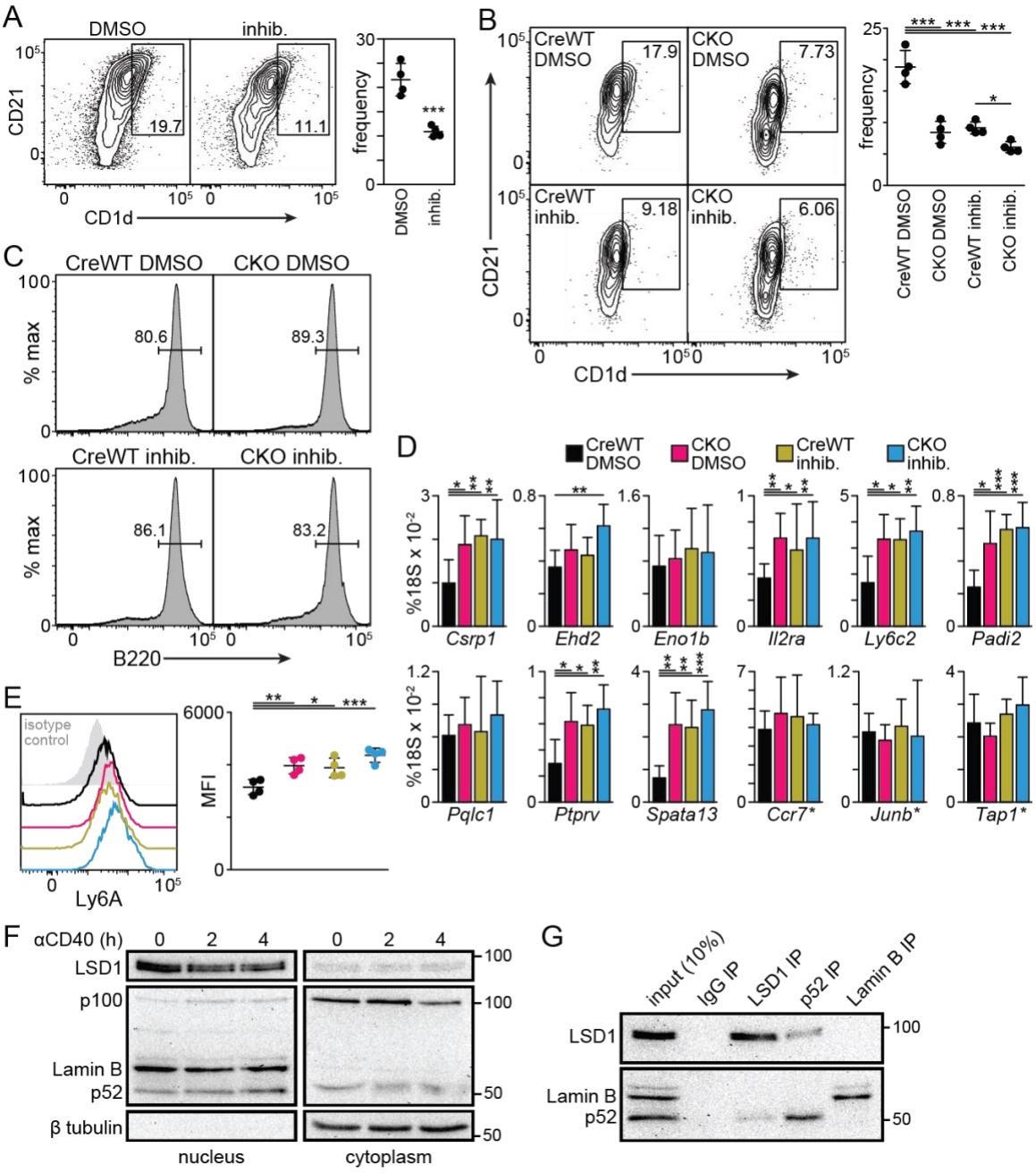


Figure 3-12 – LSD1 cooperates with non-canonical NF-κB signaling in marginal zone B cells.

(A) Flow cytometry analysis of CD21 and CD1d expression on B220^{+} C57BL/6 wild-type cells after three days of being cultured with OP9-DL1 cells in the presence of BAFF. Cells were treated with either the NF-κB inhibitor IKK-16 (inhib.) or DMSO. **(B)** Flow cytometry analysis of CD21 and CD1d expression on B220^{+} CKO or CreWT cells after three days of being cultured with OP9-DL1 cells in the presence of BAFF. Cells were treated with either the NF-κB inhibitor IKK-16 (inhib.) or DMSO. **(C)** Flow cytometry analysis of B220 expression on APC^{+} enrichments of B220-APC-stained cultures from B.

(D) RT-qPCR expression data relative to 18S expression of select genes. Canonical NF-κB target genes are denoted by *. **(E)** Flow cytometry analysis of Ly6A/ for the four populations of cells gated in part E. **(F)** Western blot of Raji cell nuclear and cytoplasmic lysates collected following zero, two, and four hours of αCD40 Ab treatment. **(G)** Co-immunoprecipitations from Raji cell nuclear extracts at four hours following αCD40 Ab treatment. All flow cytometry data are representative of two independent experiments using four to five mice per group. Error bars represent mean ± SD. Significance was determined by Student's paired two-tailed *t*-test. *P<0.05, **P<0.01, ***P<0.001.

repression by NF-κB signaling (**Fig. 3-12 D**). The six significant genes from above plus the gene *Ehd2* were significantly upregulated in CKO inhibitor cultures relative to CreWT DMSO cultures, indicating possible pathway overlap. The three conical NF-κB signaling genes were not differentially expressed in any condition, supporting that the gene dysregulation observed is due to a defect only in non-canonical NF-κB signaling. Flow cytometry was used to validate the surface expression of Ly6A, which was found to be significantly increased upon LSD1 deletion and NF-κB inhibition (**Fig. 3-12 E**).

Endogenous interaction of LSD1 with p52 was examined by co-immunoprecipitation experiments performed in the Raji human B cell line[389], from which sufficient quantities of protein could be obtained. To induce p52 nuclear translocation, Raji cells were treated with anti-CD40 Ab, and nuclear and cytoplasmic fractions were assessed (**Fig. 3-12 F**). At four hours post-treatment, nuclear p52 levels increased while its cytoplasmic precursor p100 levels decreased, indicating that anti-CD40 Ab treatment successfully stimulated p52 nuclear translocation. Co-immunoprecipitation of p52 and LSD1 was performed on Raji nuclear lysate at four days post anti-CD40 Ab treatment (**Fig. 3-12 G**). LSD1 immunoprecipitated with p52 and p52 immunoprecipitated with LSD1, indicating that the two proteins are found within the same complex. Together, these data

confirm a critical regulatory role for non-canonical NF-κB signaling and demonstrate a cooperative relationship between this signaling pathway and LSD1 in MZB development.

Chapter 4: Work in Progress

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There are multiple experiments remaining that would follow up work highlighted in chapters 2 and 3. Regarding chapter 2, LSD1-target genes can be overexpressed in *ex vivo* differentiated B cells to determine exactly which genes contribute to the phenotype. This would be performed with a lentivirus system in which 1) HEK293 cells are transfected with plasmids encoding proteins that make up lentivirus particles and an expression construct of the gene downstream of a mouse promoter that also activates a marker gene such as GFP, 2) viral particles generated by the HEK293 cells are purified, 3) naïve mouse B cells are infected *ex vivo*, 4) infected cells are fed LPS, IL-2, and IL-5 to differentiate, and 5) cultures are assayed for GFP^+ $CD138^+$ cell frequencies and numbers. Also, experiments such as ChIP-seq or CUT&RUN that assay the genome-wide landscape of LSD1 binding, as well as the LSD1 target histone modifications H3K4me1, H3K4me2, H3K9me1, and H3K9me2 in naïve B cells, activated B cells, and plasmablasts would clearly define which genes are directly regulated by LSD1. This data analyzed in conjunction with the published ChIP-seq datasets of Blimp-1, IRF4, and PU.1 in LPS-induced plasmablasts would provide further insight into which factors LSD1 interacts with to perform its gene regulatory functions. Finally, it is not well known which epigenetic modifying enzymes regulate memory B cell formation and function. The role of LSD1 during this process still needs thorough exploration.

Regarding chapter 3 and similar to chapter 2, LSD1 target genes can be overexpressed in *ex vivo* developed marginal zone B cells using the same lentivirus system. If a gene was found to be critical for *ex vivo* marginal zone B cell development, a conditional deletion mouse of that specific gene would be generated and *in vivo* marginal zone B cell development would be assayed. Also similar to chapter 2, a genome-wide

landscape of LSD1 and p52 binding, either through ChIP-seq or CUT&RUN, is needed to fully attribute LSD1 to the regulation of p52 target genes in marginal zone B cells. Genome-wide histone modification landscapes would prove that the enzymatic activity of LSD1 is key for this process.

An important project stemming from chapter 3 consists of characterizing the B-1 cell population defects observed in CKO mice. As shown in **Fig. 3-3 A-C**, LSD1-deficient CKO mice exhibit significant alterations in B-1 cell frequencies: B-1a populations are significantly decreased in the spleen and peritoneal cavity of CKO mice and there are either fewer B-1 cells or more B-2 cells in the peritoneal cavity of CKO mice. Further flow cytometry-based phenotyping experiments revealed that there is a significant increase in $CD11b^+$ $CD23^+$ B cells gated from IgM^+ B cells in the peritoneal cavity (**Fig. 4-1**). This data suggests that LSD1 may be regulating the expression of these markers in either B-2 or B-1 cells. A 2006 paper showed that if peritoneal B-2 cells were adoptively transferred into SCID mice (severe combined immune deficiency mice, or mice that lack both B cells and T cells), they upregulated CD11b and CD43 and downregulated $CD23$[390], supporting that LSD1 may be regulating B-2 cell gene expression based on peritoneal cavity molecular cues. Alternatively, this population may represent a rare developmental intermediate that accumulates due to the inability to develop without LSD1. To address the origin question, an HSC transplantation experiment can be performed by which CKO and CreWT HSCs from the fetal liver are transferred in an equal ratio into the peritoneal cavity of μMT mice (mice that lack B cells) and reconstitution frequencies are measured. If most B-1 cells are of CreWT origin and $CD11b^+$ $CD23^+$ CKO cells accumulate, then the defect observed may be due to an LSD1-dependent block in development. Also, given the decrease in B-1a

cells, CKO mice may be predisposed to poorly respond to pathogens more readily countered by B-1a cells. For example, B-1a cells are known to respond better to influenza compared to B-1b cells[391], so immunizing mice with influenza and quantifying the B-1 cell response by flow cytometry may answer this question. Overall, the proposed experiments would more accurately define the role of LSD1 in B cell development and differentiation.

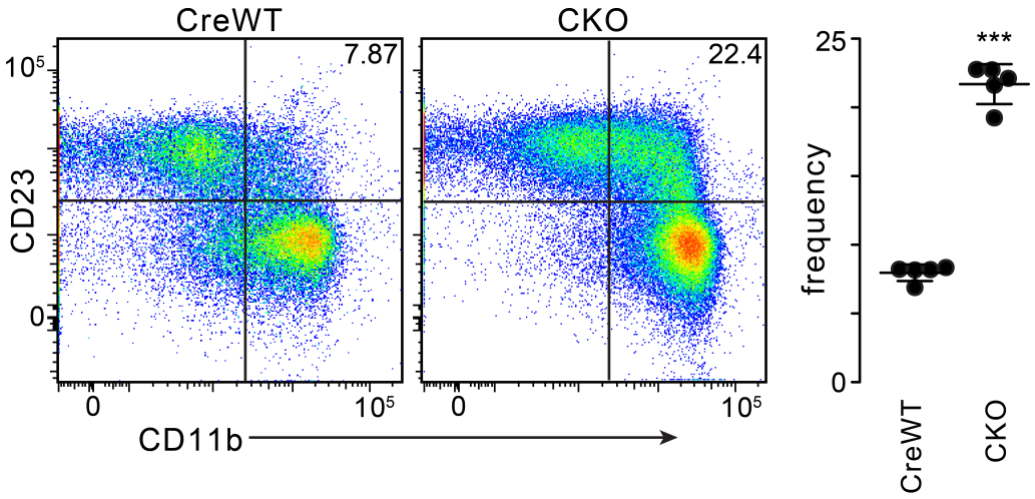

Figure 4-1 – CKO mice possess a significant expansion of peritoneal cavity $IgM^+CD23^+CD11b^+$ cells
Flow cytometry analysis of the expression of CD23 and CD11b on peritoneal cavity IgM^+ B cells in CreWT and CKO mice.

Chapter 5: Discussion

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The work presented in this thesis contributes to the scientific community in multiple ways. First, it introduces novel epigenetic and transcriptional regulatory functions of LSD1 during cellular developmental and differentiation pathways, which will be of general interest to those studying genetics and epigenetics. Second, LSD1 was shown to be critical for B cell development and differentiation, which are processes essential for adaptive immune system function. Understanding how the adaptive immune system normally functions is critical to understanding how B cell-based diseases such as certain autoimmune diseases and leukemias arise and persist. Third, this work provides high-quality transcriptomic and epigenetic sequencing data sets to the public, which will contribute to developing and completing future research projects from our lab and others.

This work defines the regulatory role of LSD1 during TI antigen-induced B cell differentiation and dissects its impact on the plasmablast epigenome and transcriptome (**Fig. 5-1**)[348]. The recently published work from the Melnick lab showed that LSD1 is also critical for TD antigen-induced B cell differentiation[80]. However, some aspects of LSD1-based regulation of B cell differentiation remain unknown. This study showed that LSD1-deficient naïve B cells exhibit diminished differentiation into short-lived PB in response to the TI antigen LPS and the TD antigen influenza virus, while the Melnick study showed that LSD1-deficient naïve B cells exhibit diminished germinal center B cell differentiation in response to the TD antigen sheep red blood cells. It is still unclear if LSD1 regulates the differentiation in response to other TI antigens such as CpG or if LSD1 regulates the differentiation of long-lived plasma cells or memory B cells following the germinal center reaction. Testing the latter will be technically challenging because of how important LSD1 is for germinal center B cell differentiation and may require the use of an inducible deletion

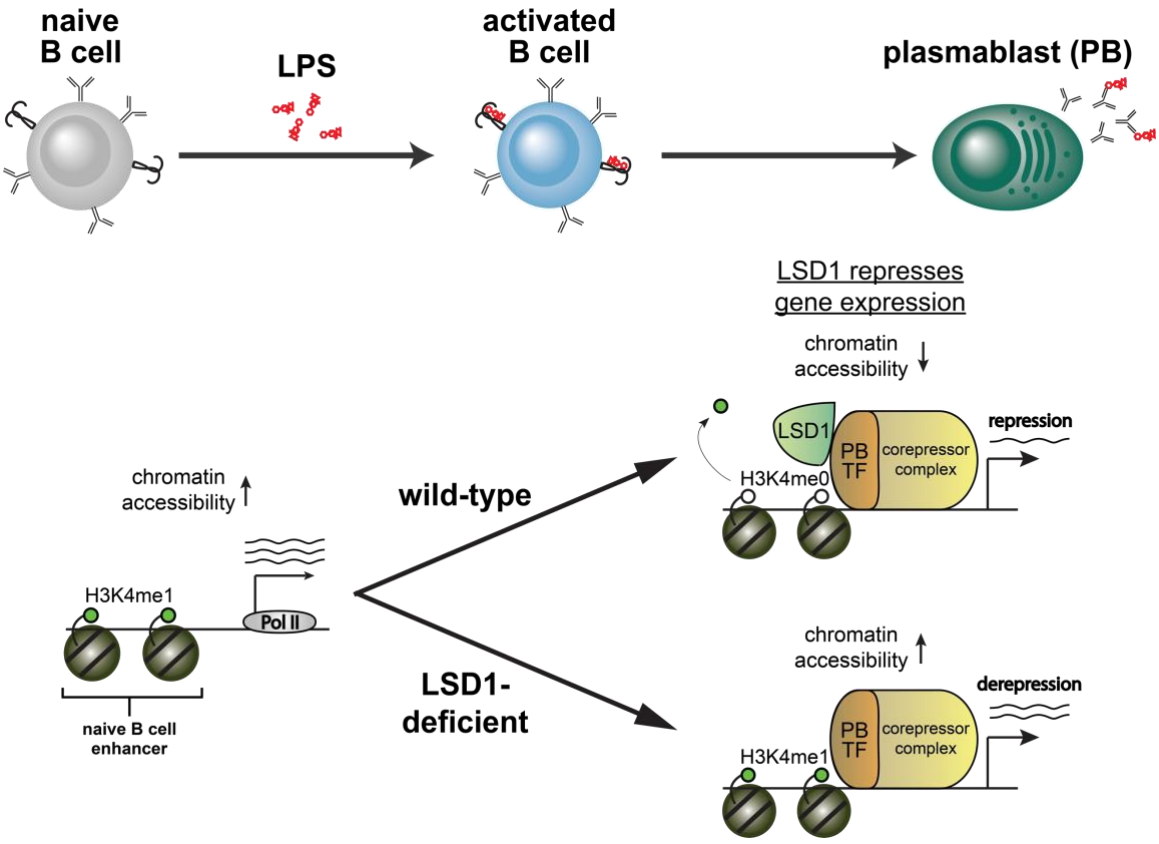

Figure 5-1 – Model for LSD1-mediated gene repression in LPS-induced plasmablasts Model illustrating how LSD1 decommissions naïve B cell enhancers in plasmablasts to facilitate gene repression. PB TF = plasmablast transcription factor.

system such as the *Rosa26*^{CreERT2} system used here. Also, it will be interesting to see if LSD1-deficient memory B cells also exhibit the same defect in differentiation as naïve B cells do.

The role of LSD1 during B cell development was also highly defined in this work (**Fig. 5-2**). LSD1 seems to be critical during splenic B cell development, but dispensable for bone marrow B cell development. However, bone marrow chimera data (**Fig. 3-4 E, G**) did indicate a slight but significant decrease in the ability of LSD1-deficient bone marrow pre-B cells to compete against LSD1-sufficient cells. This is noteworthy because a study published in 2017 using a pro-B cell line showed that LSD1 interacts with the

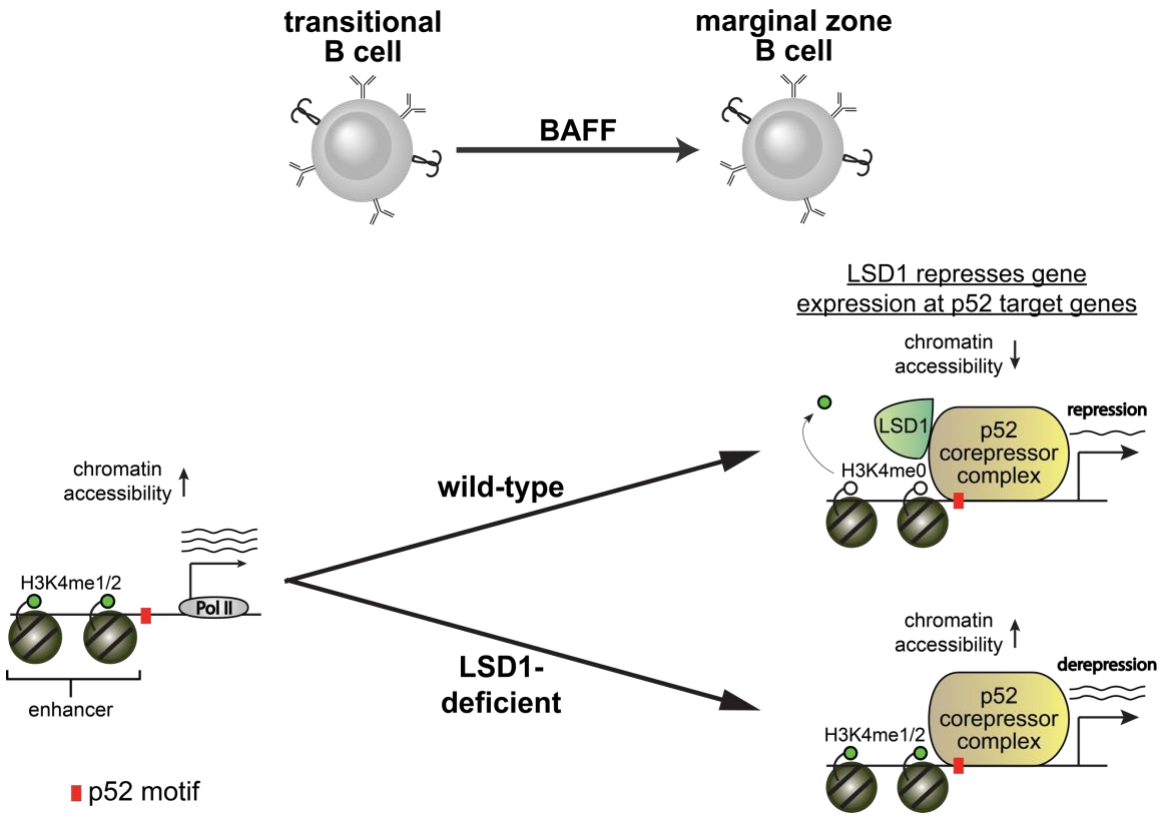

Figure 5-2 – Model for LSD1-mediated gene repression during MZB development
Model illustrating how LSD1 may be repressing gene expression during MZB development.

transcription factor STAT5a to regulate the expression of dozens of genes[392]. Together, these data suggest a possible *in vivo* role for LSD1 during the pro-B cell to pre-B cell transition. LSD1 also regulates B-1 cell development to an extent, indicated by a slight but significant expansion in B-1b cells. The mechanism for this is unclear, but it may be related to regulation of NF-κB, since canonical NF-κB is known to regulate B-1 cell development[393]. The alteration in B-1 and B-2 cell frequencies in the peritoneal cavity may indicate a decrease in B-1 cells or an increase in B-2 cells. As mentioned in chapter 4, further experiments examining the role of LSD1 during fetal B cell development are necessary to understand this phenotype.

LSD1 seems to cooperate with the transcription factors Blimp-1, PU.1, and IRF4 to mediate its regulatory affect in differentiating plasmablasts while it cooperates with the transcription factor p52 to mediate its regulatory affect in developing MZB. In both pathways, it is unclear which specific LSD1-target gene(s) causes the observed decrease in populations. In LSD1-deficient plasmablasts, dozens of cell cycle genes are downregulated with a concomitant decrease in the proliferative capacity of LSD1-deficient naïve B cells, suggesting that the decrease is at least partially due to a cell cycle defect. However, 471 genes are aberrantly upregulated in these cells, making it likely that the upregulation of one or more of these genes contributes to the phenotype as well. For example, CD300a was superinduced in LSD1-deficient PB. It has been shown CD300a negatively regulates BCR-stimulus-induced B cell proliferation[316], suggesting that overexpression in the LPS-stimulation model may dampen B cell proliferation. Unlike in LSD1-deficient plasmablasts, LSD1-deficient MZB do not exhibit any downregulation in cell cycle genes. Instead, 297 genes are aberrantly upregulated, including p52-target genes Ly6a, which regulates hematopoietic stem cell development and survival[383], and Id3, a transcription factor critical for splenic B cell development[355]. Experiments testing gene overexpression in these pathways will help deduce the exact mechanism of LSD1-based regulation.

In both plasmablast differentiation and marginal zone B cell development, LSD1 primarily repressed chromatin accessibility, providing the first piece of evidence that LSD1 is participating in H3K4me1/2 demethylation during both processes. In plasmablasts but not naïve B cells, LSD1 demethylated H3K4me1 at naïve B cell enhancers that mapped to LSD1-target genes, suggesting that LSD1 decommissioned naïve B cell enhancers in plasmablasts. Additionally, these enhancers were bound by the transcription factors PU.1,

IRF4, and Blimp-1, implying that LSD1 is recruited to these sites by these factors to perform its demethylase activity. It is unlikely that LSD1 is participating in H3K9me1/2 demethylation in plasmablasts because the small number of decreases in chromatin accessibility in LSD1-deficient plasmablasts do not map to LSD1-target genes. The chromatin accessibility data in marginal zone B cells suggested a similar enhancer decommissioning mechanism for p52, however no ChIP-seq data exists for this factor in this cell type. Additionally, cell numbers are limiting for marginal zone B cells (< 1,000,000 cells per mouse), so our lab was unable to perform ChIP-based assays on this cell type to probe histone modifications and protein binding. We are currently in the process of optimizing the CUT&RUN assay, which requires vastly lower cell input compared to ChIP-seq, so that we can assess the chromatin landscape in LSD1-sufficient and -deficient marginal zone B cells.

The most surprising finding of the entire work was that B cell-conditional deletion of LSD1 impairs MZB development but not FoB development, despite LSD1 cooperating with non-canonical NF-kB signaling. BAFF-mediated non-canonical NF-kB signaling is critical for both the development of FoB and MZB[285], suggesting that some sort of molecular mechanism restricts p52-based LSD1 function to the MZB lineage. All five NF-κB family member genes are expressed similarly in MZB and FoB, thus no NF-κB transcription factor is present only in MZB to facilitate LSD1-dependent gene regulation. Genes expressed exclusively by MZB may be influencing NF-κB-based LSD1 activity. For example, the non-canonical IKK kinase IKKε promotes gene regulatory capabilities of a p52-p65 NF-κB complex[394] and is significantly upregulated in MZB compared to FoB[347], suggesting increased p52 activity in MZB. The high expression of both *Nfkb2* and *Rela* in

eMZB (**Fig. 3-10 C**) in addition to previous work showing that both p52 and p65-based NF-κB complexes are capable of repressing genes through epigenetic mechanisms[395,396] support the possibility of a p52-p65 complex functioning with LSD1 as a transcriptional repressor during MZB development. Also, because cell signaling is known to drive cell fate bifurcation in other hematopoietic developmental pathways[397,398], it is possible that gene programs induced by NOTCH2 signaling may be influencing LSD1-based selectivity.

In summary, there are multiple unanswered questions remaining from this work. Memory B cells undergo rapid differentiation upon antigen stimulation, similar to B cells responding to TI antigens, thus examining the role of LSD1 during memory B cell differentiation seems like a logical next set of experiments. The expansion of $CD11b^+$ $CD23^+$ cells in the peritoneal cavity is exceedingly intriguing, since no other study has identified such a B cell subset, so understanding the LSD1-based defect behind this phenotype will also be prioritized. Importantly, although this work identifies LSD1 as a key epigenetic regulator of B cell development and differentiation, it does not uncover the full snapshot of how all epigenetic regulators work in sync to drive the humoral immune response. For example, the H3K4me2/3 histone demethylase JARID1C is upregulated in LPS-induced plasmablasts to a degree equal to LSD1 (**Fig. 2-1 C**), so to fully understand H3K4-based regulation of B cell differentiation, the role of JARID1C will also have to be examined. By assessing the individual and combined roles of all expressed epigenetic modifying enzymes in a biological process, a complete epigenetic network that can be exploited for therapeutic purposes will be established.

The epigenetic reprogramming of lymphocytes during development is crucial for proper immune system formation and function[399]. Developing B cells in the bone marrow

and differentiating B cells in response to both TI and TD antigen exhibit distinct patterns of chromatin accessibility and histone modifications[295,296,400], and this work confirms previous work and demonstrates that the epigenome remains dynamic throughout splenic B cell development as well. Therapeutic targeting of histone modifying enzymes is used to treat numerous hematopoietic malignancies[401,402], and the data presented here support that malignancies arising from splenic B cell development, such as marginal zone B cell lymphomas[403], as well as those arising from B cell differentiation, such as multiple myeloma[404], can be targeted as well. Overall, this work defines LSD1 as a critical epigenetic and transcriptional regulator of splenic B cell development and plasmablast differentiation, identifies cooperation between LSD1 and multiple B cell transcription factors, and expands our knowledge of the epigenetic regulation of the adaptive immune system.

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