Regulation of hematopoiesis and B-lymphocyte development by histone deubiquitinases

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ABSTRACT

Hematopoiesis, or the production of all blood cells from a rare population of hematopoietic stem cells (HSCs), is a tightly regulated process that replenishes our blood and immune systems throughout life. In the pool of blood cells, B cells are crucial in the adaptive immune response for their secretions of specific antibodies targeting foreign pathogens. B cells arise from HSCs within the bone marrow following multiple steps of cell differentiation and complete their maturation in secondary lymphoid organs with help from T cells and antigen stimulation. Dysregulation during B-cell development may result in immunodeficiencies, autoimmune diseases, and malignancies.

Chromatin-modifying enzymes are important in hematopoiesis and B-cell physiology as loss-of-function mutations in these proteins often associate with hematologic abnormalities. Among the chromatin modifications, (de)ubiquitination of histones remains one of the least characterized modifications despite its early discovery. The process of histone (de)ubiquitination participates in the regulation of gene transcription, DNA repair, and cell cycle progression, all of which are essential during blood cell differentiation and development.

This thesis characterizes the functions of three histone deubiquitinating enzymes in the regulation of hematopoiesis using mouse models. Within the B-cell compartment, deubiquitinase BAP1 regulates cellular development and the transcriptional program of cell cycle progression in pre-B cells. In the context of c-MYC-driven B-cell lymphoma, loss of the deubiquitinase MYSM1 protects mice against the oncogenic activity of c-MYC. On the other hand, for the normal functions of HSCs and the development of lymphocytes, deubiquitinase USP44 is dispensable, possibly due to functional redundancy. Together, our findings unravel the significance of histone deubiquitinating enzymes in transcriptional regulation of hematopoiesis and may contribute to the development of improved therapies for c-MYC-driven hematologic malignancies.

RÉSUMÉ

L'hématopoïèse, ou la production de toutes les cellules sanguines à partir d'une population rare de cellules souches hématopoïétiques (CSH), est un processus étroitement réglementé qui reconstitue notre sang et notre système immunitaire tout au long de la vie. Dans le pool de cellules sanguines matures, les cellules B jouent un rôle crucial dans la réponse immunitaire adaptative pour leurs sécrétions d'anticorps spécifiques ciblant des pathogènes étrangers. Les cellules B proviennent de CSH dans la moelle osseuse après plusieurs étapes de différenciation cellulaire et achèvent leur maturation dans les organes lymphoïdes secondaires à l'aide des cellules T et de la stimulation antigénique. Une dérégulation pendant le développement des cellules B peut entraîner des déficits immunitaires, des maladies auto-immunes et des tumeurs malignes.

Les enzymes de modification de la chromatine sont importantes dans l'hématopoïèse et la physiologie des cellules B car les mutations de perte de fonction de ces protéines s'associent souvent à des anomalies hématologiques. Parmi les modifications de la chromatine, la (dés) ubiquitination des histones reste l'une des modifications les moins caractérisées malgré sa découverte précoce. Le processus de (dés) ubiquitination des histones participe à la régulation de la transcription des gènes, de la réparation de l'ADN et de la progression du cycle cellulaire, qui sont tous essentiels au cours de la différenciation et du développement des cellules sanguines.

Cette thèse caractérise les fonctions de trois enzymes de désubiquitination des histones dans la régulation de l'hématopoïèse à l'aide de modèles murins. Dans le compartiment des cellules B, l'enzyme de désubiquitination BAP1 régule le développement cellulaire et le programme transcriptionnel de la progression du cycle cellulaire dans les cellules pré-B. Dans le contexte du lymphome à cellules B induit par c-MYC, la perte de l'enzyme de désubiquitination MYSM1 protège les souris contre l'activité oncogène du c-MYC. D'autre part, pour les fonctions normales

des CSH et le développement des lymphocytes, l'enzyme de désubiquitination USP44 est inutile, probablement en raison d'une redondance fonctionnelle. Ensemble, nos résultats démontrent l'importance des enzymes de désubiquitination des histones dans la régulation transcriptionnelle de l'hématopoïèse et peuvent contribuer au développement de thérapies améliorées pour les hémopathies malignes induites par le c-MYC.

PREFACE

This thesis is written in the traditional format. The work presented in this thesis consists of experiments I have performed for the following first-author publications and manuscripts:

Chapter 3.1: **Yun Hsiao Lin**, Yue Liang, HanChen Wang, Lin Tze Tung, Michael Förster, Poorani Ganesh Subramani, Javier M. Di Noia, Simon Clare, David Langlais, Anastasia Nijnik. (2021) Regulation of B Lymphocyte Development by Histone H2A Deubiquitinase BAP1. *Frontiers in Immunology*. **12**:626418. doi: 10.3389/fimmu.2021.626418. (1)

Chapter 3.2: **Yun Hsiao Lin**, HanChen Wang, Amanda Fiore, Michael Förster, Lin Tze Tung, Jad I Belle, Francis Robert, Jerry Pelletier, David Langlais, Anastasia Nijnik. Loss of MYSM1 inhibits the oncogenic activity of cMYC in B cell lymphoma. (Manuscript accepted in the *Journal of Cellular and Molecular Medicine*)

Chapter 3.3: **Yun Hsiao Lin**, Michael Forster, Yue Liang, Mansen Yu, HanChen Wang, Francis Robert, David Langlais, Jerry Pelletier, Simon Clare, Anastasia Nijnik. (2019) USP44 is dispensable for normal hematopoietic stem cell function, lymphocyte development, and B-cell-mediated immune response in a mouse model. *Experimental Hematology*. **72**, 1-8. doi: 10.1016/j.exphem.2019.01.001. (2)

CONTRIBUTION OF AUTHORS

Chapters 1 and 4: The writing was kindly reviewed by Dr. Anastasia Nijnik.

Chapter 3.1: Experiments were designed by Dr. Anastasia Nijnik, **Yun Hsiao Lin**, Dr. David Langlais, and Dr. Michael Förster, and the experimental work was carried out and data acquired by **Yun Hsiao Lin**, with assistance from Lin Tze Tung, Dr. Michael Förster, and Dr. Anastasia Nijnik. I bred most of the *Bap1*$^{fl/fl}$ mb1-*Cre*, *Bap1*$^{fl/+}$ mb1-*Cre*, and control *Bap1*$^{fl/+}$ mice. Bioinformatics data analysis was performed by HanChen Wang with the supervision of Dr. David Langlais. Dr. Javier M. Di Noia and Poorani Ganesh Subramani provided scientific advice and input on data analyses and interpretation. Dr. Simon Clare provided *Bap1*$^{tm1c(EUCOMM)Hmgu}$ mice, genotyping protocols, and scientific advice. The manuscript was written by Dr. Anastasia Nijnik, **Yun Hsiao Lin**, Yue Liang, and HanChen Wang.

Chapter 3.2: The experiments in the Results section were performed by **Yun Hsiao Lin**, Amanda Fiore, and Dr. Michael Förster. I bred most of the *Eμ-Myc Mysm1*$^{fl/fl}$ *Cre*^{ERT2} and control *Eμ-Myc Mysm1*$^{fl/+}$ *Cre*^{ERT2} mice, harvested their tumours, irradiated wild-type recipient mice, performed intraperitoneal injections of tamoxifen/ corn oil into transplanted recipients, and monitored mouse survival. I bred most of the *Eμ-Myc Mysm1*$^{-/-}$ and control *Eμ-Myc Mysm1*$^{+/-}$ mice, harvested and cultured their tumours, collected them for cell sorting, extracted RNA, and performed protein synthesis assay and intracellular flow cytometry experiments. In the Appendix section, bioinformatics data analysis was performed by HanChen Wang, Jad I Belle, and Dr. David Langlais. The manuscript was written by Dr. Anastasia Nijnik, **Yun Hsiao Lin**, and HanChen

Wang. The experiments were designed by Dr. Anastasia Nijnik and Dr. David Langlais, with the advice of Dr. Francis Robert and Dr. Jerry Pelletier. The work was supervised by Dr. Anastasia Nijnik, Dr. David Langlais, Dr. Francis Robert, and Dr. Jerry Pelletier.

Chapter 3.3: The experiments were designed by Dr. Anastasia Nijnik, **Yun Hsiao Lin**, and Dr. Michael Forster and performed by **Yun Hsiao Lin**, Dr. Michael Forster, Yue Liang, Mansen Yu, and HanChen Wang. I bred most of the *Usp44*$^{-/-}$, *Usp44*$^{+/-}$, and control *Usp44*$^{+/+}$ mice. With tremendous help from Dr. Anastasia Nijnik and Dr. Michael Forster in mouse phenotyping/ flow cytometry, I performed the experiments in Figures 3.3.1F, 3.3.2-3.3.4, 3.3.5A-C, 3.3.5E-F, 3.3.5I, 3.3.6C. Dr. Michael Forster performed the experiment for Figure 3.3.5D, 3.3.5G. Yue Liang performed the experiments for Figure 3.3.1A-E. I immunized *Usp44*$^{-/-}$, *Usp44*$^{+/-}$, and control *Usp44*$^{+/+}$ mice and collected serum via cardiac puncture, and Mansen Yu performed ELISA for Figure 3.3.5H. HanChen Wang performed the analysis for Figure 3.3.6A-B. The data were analyzed, and the manuscript prepared by Dr. Anastasia Nijnik and **Yun Hsiao Lin**. Dr. Simon Clare provided the mouse line and scientific advice; Dr. Anastasia Nijnik, Dr. David Langlais, Dr. Jerry Pelletier, and Dr. Simon Clare supervised the work. Dr. Francis Robert and Dr. Jerry Pelletier provided the expertise with the analysis of Eμ-Myc B-cell lymphoma mouse model.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

This thesis furthers our understanding of the roles of three histone deubiquitinases in the hematopoietic system. Our main findings and contribution to original knowledge are summarized:

- We generated a B-cell-specific *Bap1*-knockout mouse model and found a cell-intrinsic role of BAP1 in B-cell development. We characterized BAP1 as a transcriptional regulator of cell cycle progression in pre-B cells during B-cell development.

- We found a beneficial effect of *Mysm1*-loss in the survival of Eμ-Myc mice of B-cell lymphoma. We demonstrated that deletion of *Mysm1* in transplanted Eμ-Myc tumour cells prolonged the survival of recipient mice, suggesting disease remission. We showed reduced expression of ribosomal protein genes, decreased protein synthesis rate, and elevated p53 protein level in *Mysm1*-deficient Eμ-Myc tumour cells.

- We investigated the hematopoietic and immune systems of *Usp44*-knockout mice and established that USP44 is nonessential for hematopoietic stem cell function, lymphocyte development, B-cell-mediated immunity, and B-cell lymphoma progression. We suggested that USP44 may be functionally redundant with other USP-family proteins.

OTHER CONTRIBUTIONS NOT INCLUDED IN THIS THESIS

In addition, I have had the opportunity to contribute to the following publications that are not included in this thesis:

Jad I Belle*, HanChen Wang*, Amanda Fiore, Jessica C Petrov, **Yun Hsiao Lin**, Chu-Han Feng, Thi Tuyet Mai Nguyen, Jacky Tung, Philippe M Campeau, Uta Behrends, Theresa Brunet, Gloria Sarah Leszinski, Philippe Gros, David Langlais, Anastasia Nijnik. (2020) MYSM1 maintains ribosomal protein gene expression in hematopoietic stem cells to prevent hematopoietic dysfunction. *JCI Insight.* **5**(13):e125690. doi: 10.1172/jci.insight.125690.

Amanda Fiore, Yue Liang, **Yun Hsiao Lin**, Jacky Tung, HanChen Wang, David Langlais, Anastasia Nijnik. (2020) Deubiquitinase MYSM1 in the Hematopoietic System and beyond: A Current Review. *International Journal of Molecular Sciences.* **21**(8):3007. doi: 10.3390/ijms21083007.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Most of our blood and immune cells originate from a rare population of hematopoietic stem cells (HSCs) within the bone marrow, following the process of cell proliferation and differentiation known as hematopoiesis (3). Further steps of cell activation, proliferation, and differentiation are required for the immune response against infection (3-6). Dysregulation of the mechanisms that govern gene transcription, cell cycle progression, and genomic stability in these blood and immune cells frequently associates with immunodeficiency, bone marrow failure, and cancer (4, 7, 8).

"B cells are mediators of the humoral immune response, with crucial roles in the defense against infections, anti-tumour immunity, allergic response, and autoimmune disorders (4, 9, 10). B cell development takes place in the bone marrow and proceeds through multiple well-characterized developmental stages, guided by the progressive activation of B cell specific transcriptional programs and sequential rearrangement of the immunoglobulin loci. The transcriptional programs of B cell differentiation are controlled by a network of transcription factors E2A, EBF1, PAX5, IKAROS, BCL11A, FOXO1, IRF4, IRF8 and others that work in concert with many histone modifying enzymes and chromatin remodeling complexes (11-14)" (1).

"Monoubiquitination of histone H2A (H2AK119ub) is a highly abundant histone modification, associated with gene silencing (15, 16). It is primarily deposited on chromatin by the RING1B subunit of the Polycomb repressive complex 1 (PRC1), which acts as a major epigenetic regulator of cell identity, differentiation, and development (16-18). The role of PRC1 in B cell development and lymphomagenesis is well established (17, 19, 20), and the loss of PRC1

complex components in conditional knockout mouse models impairs B cell differentiation and disrupts B cell specific transcriptional programs (21-26). Several deubiquitinases (DUBs) were shown to reverse the monoubiquitination of histone H2A, including BAP1, USP16, MYSM1, USP44, and others (15, 27). However, the role of histone DUBs in B cell development and lymphomagenesis is less well understood" (1).

1.2 Introduction to Hematopoiesis and B-cell Lymphopoiesis

1.2.1 Hematopoiesis

Hematopoiesis is the production of blood cells in our body (3). This process occurs continually throughout life and originates from rare hematopoietic stem cells (HSCs) (3). HSCs are first observed in the aorta-gonad-mesonephros region of an embryo, then in the liver of a developing fetus, and ultimately in the bone marrow, where they reside just before birth (3, 4, 28-30). These multipotent cells have the ability to self-renew and to differentiate into all mature blood cells, replenishing our blood system throughout life (3). After rounds of cell division, multipotent HSCs generate cells of the myeloid lineage (monocytes, platelet-producing megakaryocytes, red blood cells, and granulocytes), cells of the lymphoid lineage (B cells, T cells, natural killer cells, and innate lymphoid cells), as well as cells of either lineages (dendritic cells) (3, 4, 31, 32).

1.2.2 The Immune Response

Our blood cells cooperate in the immune response against foreign particles (33). The immune system defends through an immediate, nonspecific innate response, followed by a delayed, but targeted and long-lasting, adaptive response, with the goal of removing invaders and acquiring immunological memory (33). The innate immune response is the first line of defense, consisting of physical barriers (e.g., skin and mucous membranes), chemical barriers (e.g., antimicrobial peptides that kill bacteria), and phagocytic cells (e.g., macrophages, monocytes, and neutrophils) (34). Tissue macrophages are long-lived phagocytes found throughout the body, commonly near sites of infection; they uptake pathogens and secrete chemotactic cytokines (chemokines) to rapidly attract phagocytic neutrophils in large quantities (33). Neutrophils are short-lived and also

engulf pathogens (33). Together with macrophages, neutrophils release harmful intracellular enzymes to break down bacterial cell walls and proteins; these immune cells also increase oxygen consumption and produce toxic reactive oxygen species during respiratory burst to kill the engulfed pathogen (33). Macrophages generally survive and continue to search for pathogens, while neutrophils usually die and form neutrophil extracellular traps of DNA, histones, and proteins, which bind and destroy pathogens (33, 34). Dendritic cells connect the innate with the adaptive immune response (33). Dendritic cells are specialized phagocytes that bring pieces of the intruder on their cell surface from the site of infection into nearby secondary lymphoid organs to present to T cells (33). The adaptive immune response consists of T cells and antibody-producing B cells that specifically attack the antigen and form memory T cells and memory B cells, which remain in the host and are able to mount a faster and stronger attack following pathogen re-exposure (33).

1.2.3 B Lymphocytes of the Adaptive Immune Response

B lymphocytes, or B cells, are produced from the lymphoid lineage of hematopoiesis and participate in the adaptive immune response (3, 4). B cells arise from HSCs in the bone marrow through multiple well-defined stages, with tight regulation every step of the differentiation process to ensure proper recombination of the immunoglobulin chains and successful assembly of the surface B-cell receptor (3, 4). Subsequently, B cells mature in secondary lymphoid organs in response to antigen and T-cell stimulation to become germinal centre B cells, plasma cells, or memory B cells (4). Effector B cells are key players in the adaptive immune response with the production of pathogen-fighting antibodies (4). By providing the antibody molecules to neutralize toxins, activate the complement cascade, and enhance engulfment of foreign particles, B cells play

an important role in the protection against pathogens, the allergic response, and autoimmune disorders (4).

1.2.4 Early B-cell Development

Within the bone marrow, daughter cells of HSCs differentiate into lymphoid-primed multipotent progenitors (LMPPs) that produce common lymphoid progenitors (CLPs), which can give rise to lymphoid cells (4). B-cell development continues to progress from pre-pro-B cells to pro-B cells, to pre-B cells, then to immature B cells, categorized by the differential expression of immunoglobulin genes and modulated by factors produced in the bone marrow microenvironment (4, 6). Pro-B cells recombine the immunoglobulin heavy chain variable (V_H), diversity (D), and joining (J_H) gene segments (4-6). Pre-B cells pair the successfully recombined heavy chain with the surrogate light chain, enabling production of a pre-B cell receptor on the cell surface (4-6). Pre-B cells undergo several rounds of cell division before rearranging the light chain V_L and J_L genes (4-6). Completion of the process creates the conventional immunoglobulin IgM molecule, which acts as a B-cell receptor on the cell surface, denoting the cells as immature B cells (4-6). Immature B cells emerge from the bone marrow, enter the bloodstream as transitional B cells, and migrate into secondary lymphoid organs, such as the spleen and lymph nodes (5).

1.2.5 Peripheral B-cell Development

Through alternative splicing of the constant domain, transitional B cells are able to express both IgM and IgD molecules on the cell surface (4). Transitional B cells may develop into marginal zone B cells, which reside in marginal zones within the spleen and participate in the first line of defense against blood-borne pathogens, generally without the help of T cells (4, 5). On the other

hand, transitional B cells may also develop into follicular (mature) B cells, which are located within follicles of secondary lymphoid organs and take part in the highly specific, antibody-mediated adaptive immune response (4, 5).

1.2.6 B-cell Activation

Upon recognition of the cognate antigen via surface Ig receptor and receipt of cognate T-cell-dependent stimulation, follicular B cells become activated and undergo further maturation within the microenvironment (5, 35). The follicle develops into a germinal centre (GC) that is structurally characterized by a dark zone and a light zone (36). The GC dark zone is where actively proliferating GC B cells engage in somatic hypermutation, initiated by the activation-induced cytidine deaminase (AID) enzyme inducing nucleotide changes in the antigen-recognizing Ig variable domains and potentially producing receptors with modified affinity (4-6, 35, 36). Arising from the dark zone, B-cell clones, each expressing a variation of the specific B-cell receptor, enter the GC light zone region, where they re-encounter antigen, now presented on follicular dendritic cells, and survey for binding efficacy (4, 5, 36). GC B cells are allowed to cycle back into the dark zone and repeat the process of somatic hypermutation (4, 36). GC B cells with higher affinity receptors bind more competitively to antigen and are more likely to survive; the remaining GC B cells with lower affinity variants of the antigen-binding receptor die through apoptosis (4, 35). In the light zone, GC B cells also receive signals from follicular helper T cells, aiding in class switch recombination to enhance effector functions and promoting differentiation into high-affinity antibody-secreting plasma cells or memory B cells (4-6, 36, 37).

1.2.7 B-cell Carcinogenesis

Errors during B-cell maturation and activation may give rise to malignant transformation of B cells (36). In particular, B cells are the most vulnerable during Ig rearrangement, somatic hypermutation, and class switch recombination, during which B cells activate enzymes that introduce DNA double-strand breaks and mutations (36). In the worst-case scenario, accumulation of unfixed mistakes at non-Ig (off-target) regions, such as an abnormal chromosomal translocation, results in oncogene activation in B cells (4, 6, 38). For instance, in the case of Burkitt's lymphoma, the *c-Myc* (or *Myc*) gene is translocated and placed under the control of the immunoglobulin heavy chain enhancer region (noted as IgH-*Myc*), resulting in constitutive transcription of this oncogene and uncontrollable growth and proliferation of affected B cells (4, 36, 38). Similarly, in the case of follicular lymphoma, chromosomal translocation positions the *Bcl2* (B-cell lymphoma 2) gene under the control of IgH locus (IgH-*Bcl2*), leading to an overexpression of BCL2 in affected B cells and an anti-apoptotic outcome (36, 38). Both of these lymphoma subtypes are classic examples of detrimental outcome as a consequence of imprecise shuffling of genetic material, and they showcase the importance of tight regulation of DNA damage response in B-cell physiology.

1.2.8 Normal Counterpart of B-cell Malignancies

The World Health Organization (WHO) classification categorizes B-cell neoplasms based on histology, chromosomal aberrations, cell surface protein expression, and many other features (39). Each subtype of B-cell neoplasm also shares similarities with a normal B cell at a certain stage of development, known as its "normal B cell counterpart," although the counterpart may not necessarily be the cellular origin of pathogenesis (36, 38). Resemblance of B-cell neoplasms with their normal counterpart is determined mainly by the mutation status of the immunoglobulin

variable (IgV) domain and by their cellular gene expression profile (38). Pre-germinal centre mature B cell, which has unmutated IgV, is the counterpart of most mantle cell lymphoma and small lymphocytic lymphoma; germinal centre B cell, which has mutated Ig genes, is the counterpart of most follicular lymphoma, Burkitt's lymphoma, and germinal centre B-cell like (GCB) diffuse large B-cell lymphoma (DLBCL); post-germinal centre marginal zone B cell is the counterpart of mucosa-associated lymphoid tissue (MALT) lymphoma; post-germinal centre plasmablast is the counterpart of activated B-cell like (ABC) DLBCL; and post-germinal centre memory B cell, with mutated Ig genes and class switching, is the counterpart of most hairy cell leukemia (36, 38).

1.2.9 B-cell Malignancies Driven by Abnormal c-MYC Expression

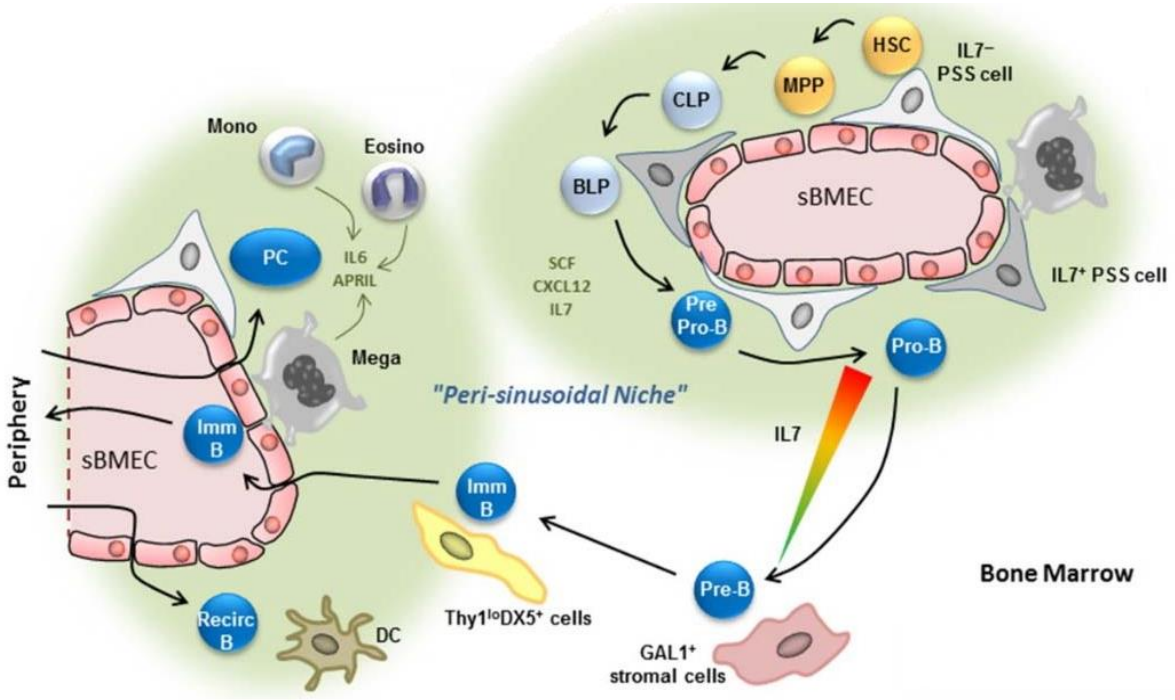
Different subtypes of B-cell neoplasm may be driven by the same transcriptional networks. One of the most commonly dysregulated pathways in cancer and hematologic malignancies involves the *c-Myc* oncogene, which encodes c-MYC protein (36, 40). MYC is an important transcription factor that regulates vital cellular functions, such as growth, proliferation, ribosome production, and apoptosis (40-42). The c-MYC protein heterodimerizes with its partner MAX and binds DNA as a c-MYC/MAX complex onto E-box regions, activating transcription of target genes (40-42). Aberrant overexpression of c-MYC, as a result of *c-Myc* rearrangement or amplification, is a common feature of non-Hodgkin lymphoma in pediatric and young adult patients, occurring in Burkitt's lymphoma (~80% of cases) and DLBCL (~5-14% of cases, correlating with inferior outcome) (42, 43). Since c-MYC-dysregulated tumours require the continuous supply of c-MYC for cell survival, c-MYC is an appealing target for anticancer therapy; however, c-MYC proves to be a difficult drug target because of the absence of a ligand-binding active site in the protein (40,

41, 44-46). Current approaches targeting c-MYC include inhibiting the transcription or translation of *c-Myc*, interrupting the heterodimerization or DNA-binding of c-MYC/MAX, and destabilizing the c-MYC protein (40, 41, 44). As the development of the most effective *c-Myc*-targeting strategy serves to be clinically impactful not only for c-MYC-overexpressing hematologic malignancies but also for a wide variety of c-MYC-deregulated cancers, this field of research is extensively studied and actively advancing (41).

1.3 Regulation of Hematopoiesis and B-cell Lymphopoiesis

1.3.1 Extrinsic Cues Within the Microenvironment

Figure 1.3.1 Bone marrow niches for B cells.



HSC: hematopoietic stem cell; MPP: multipotent progenitor; CLP: common lymphoid progenitor; BLP: B lymphoid progenitor; Imm B: immature B cell; Recirc B: recirculating B cell; DC: dendritic cell; PC: plasma cell; Mono: monocyte; Eosino: eosinophil; Mega: megakaryocyte; sBMEC: sinusoidal bone marrow endothelial cell; PSS cell: peri-sinusoidal stromal cell.

This figure is adapted from Aurrand-Lions M and Mancini SJC, 2018 (47).

1.3.1.1 Lymphoid Progenitors

CLPs likely reside in the perisinusoidal regions of the bone marrow, requiring C-X-C motif chemokine 12 (CXCL12) for development of the B cell lineage-committed CLP fraction (B lymphoid progenitors; BLPs) (47, 48). CXCL12 guides CLPs near interleukin-7 (IL-7)-producing cells, which provide IL-7 and IL-7 receptor signalling for B-cell lymphopoiesis (48). IL-7 modulates the levels of B-cell-specific transcription factor Early B-cell Factor 1 (EBF1), bestowing B-cell potential on CLPs (49).

1.3.1.2 Pre-pro-B Cells

Pre-pro-B cells are also proposed to be in the perisinusoidal niche, in the vicinity of stromal cells that produce high levels of CXCL12 for pre-pro-B cell development (47, 50). Pre-pro-B cells likely migrate from CXCL12-high niche towards IL-7-high niche for cell proliferation and differentiation into pro-B cells (51-53).

1.3.1.3 Pro-B Cells

Similar to pre-pro-B cells, pro-B cells are hypothesized to be in the perisinusoidal niche, near perisinusoidal stromal (PSS) cells that co-express CXCL12 and IL-7 and bone marrow endothelial cells that express IL-7 in low levels (47, 48). CXCL12 is important for the retention of pro-B cells in the bone marrow by sustaining the adhesion of α4β1 integrins (very late antigen-4; VLA-4) on pro-B cells to vascular cell adhesion molecule 1 (VCAM-1) on stromal cells via prolonged activation of focal adhesion kinase (FAK) (54-56). Pro-B cells are mainly stationary and require high levels of IL-7 for pro-B cell proliferation, glycolytic growth, and survival (51, 57-62). Signalling through IL-7R also promotes the silencing of premature immunoglobulin light chain

recombination (58, 61). In the pro-B cell stage, the recombination-activating gene (RAG) enzyme is activated to perform V_HDJ_H recombination of the immunoglobulin heavy chain (4-6).

1.3.1.4 Pre-B Cells

Pro-B cells differentiate into large pre-B cells (5, 6). Large pre-B cells pair the rearranged immunoglobulin heavy chain with the surrogate light chain, consisting of λ5 and VpreB proteins, and express them on the surface as the pre-B cell receptor (pre-BCR) (5, 6). Expression of pre-BCR enables selection and expansion of cells with functional immunoglobulin heavy chains (47). Large pre-B cells undergo a proliferative phase of cell division that is dependent on IL-7R signalling (51, 63). Pre-BCR signalling promotes the upregulation of CXCR4 and downregulation of FAK/VLA-4, allowing large pre-B cells to move towards CXCL12-producing stromal cells (57, 64). This chemoattraction pulls large pre-B cells away from the IL-7 gradient, escaping from IL-7R signalling (57).

Pre-BCR signalling then promotes light chain recombination and cell survival, while inhibiting cell proliferation (58, 65). Signalling through the pre-BCR may be ligand-dependent or ligand-independent, both of which utilize the extra loop of λ5 protein (47). Tonic signalling is proposed to occur when the extra loop of λ5 and the extra loop of VpreB interact with each other, or when the extra loop of λ5 interacts with a glycosylated chain on the immunoglobulin heavy chain constant region, leading to pre-BCR self-aggregation and downstream cascade in the absence of ligand-engagement (47, 66, 67). Ligand-dependent pre-BCR signalling is supported by stromal cells. Stromal cells participate with heparan sulfate proteoglycans on their cell surface and secreted galectin-1 (GAL1), which both are able to bind the extra loop of λ5 (68-70). Furthermore, GAL1 also interacts with integrins on pre-B cells that are associated with ligands on stromal cells, creating

a lattice that enhances pre-BCR clustering and signalling for pre-B cell development (71, 72). Consistently, large pre-B cells are found in close association with non-perisinusoidal stromal cells expressing galectin-1 (GAL1), requiring GAL1 and low levels of IL-7 for cell development (73).

Large pre-B cells move on to become small pre-B cells. In this stage, cells are in close proximity to CXCL12 and are directed by the CXCL12-CXCR4 axis, which downregulates pre-BCR expression and takes over the cellular programs started by pre-BCR signalling to carry on promoting light chain recombination and repressing cell proliferation (51, 64). Small pre-B cells reactivate RAG enzymes to rearrange immunoglobulin light chain V_L and J_L genes (V_LJ_L) (5, 6).

1.3.1.5 Immature B Cells

Immature B cells are characterized by the surface expression of immunoglobulin IgM molecules, which are made up of successfully recombined heavy and light chains and serve as B-cell receptors together with the signalling transduction moiety Igα and Igβ (4, 33). The B-cell receptors on immature B cells are then examined for their tolerance to self, in a process known as negative selection, to avoid self-reactive immature B cells (33). Autoreactive immature B cells upregulate CXCR4 expression and are not as capable of egressing; they stay in the bone marrow and are protected from apoptosis in the presence of cells expressing DX5 but low levels of Thy1 (74-76). These Thy1-dull DX5-positive bone marrow cells also promote *Rag* expression in immature B cells for rearrangement of the light chain genes and receptor editing (75, 76). On the other hand, non-autoreactive immature B cells downregulate CXCR4 and have reduced ability to adhere to VCAM-1 adhesion protein, promoting their exit to the periphery (56, 74). Immature B cells enter the blood and travel to secondary lymphoid organs as transitional B cells (5).

1.3.1.6 Transitional, Marginal Zone, and Follicular B Cells

Transitional B cells within the spleen differentiate into mature B cells, including marginal zone B cells and follicular B cells, with their cell fate potentially determined by the strength of B-cell receptor (BCR) signalling and their cell survival dependent on key receptor signalling pathways (77, 78). In immunoglobulin heavy chain transgenic mice, phosphorylcholine-specific immature B cells particularly gave rise to marginal zone B cells (77, 79). As phosphorylcholine is a component of the cell wall of encapsulated bacteria that are present in gut microbiota, this finding suggests that slightly self-reactive cells may have a higher chance of becoming marginal zone B cells (77, 79). Peripheral B cells rely on continuous BCR signalling and B-cell activating factor receptor (BAFF-R) signalling for cell survival (77). Reduced BCR signalling resulted in the loss of peripheral B cells and impaired development of follicular B cells, without influencing differentiation of marginal zone B cells, whereas deletion of the *Tnfsf13b* gene encoding BAFF in mice led to a depletion of marginal zone and follicular B cells (77, 78, 80, 81). Marginal zone B cells further need NOTCH signalling for proper cell development (77, 78). Depletion of proteins in the NOTCH signalling pathway abolished marginal zone B cells, but not follicular B cells (78, 82, 83). Marginal zone B cells express high levels of NOTCH2, and cells within the red pulp of the spleen express the NOTCH ligand, Delta-like 1 (DLL1) (78, 83, 84). This receptor-ligand interaction has been proposed to be involved in positioning of marginal zone B cells (78, 83, 84).

1.3.1.7 Mature B Cells in the Bone Marrow

Mature B cells and plasmablasts also circulate back into the bone marrow and reside in perisinusoidal regions, requiring the CXCL12/ CXCR4 axis for homing (55, 85, 86). Homing of these mature B cells in the bone marrow allows protection of the developing hematopoietic and

immune cells from pathogens; these mature B cells are also supported by hematopoietic cells through secretions of survival cues (47, 86). For instance, mature recirculating B cells are located close to clusters of dendritic cells, which produce the necessary macrophage migration-inhibitory factor (MIF) for their survival, whereas long-lived (memory) plasma cells within the bone marrow depend on a proliferation-inducing ligand (APRIL) and IL-6 produced by monocytes, eosinophils, and megakaryocytes for survival (87-90). Long-lived plasma cells then anchor onto CXCL12-expressing stromal cells and become immobile in the bone marrow (91, 92).

1.3.2 Transcriptional Regulation

The process of hematopoietic development is tightly regulated by a complex network of transcription factors (30). Through loss- and gain-of-function methods, studies have revealed the functional involvement of multiple key transcription factors at specific stages of development (30). Using chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq), studies have also elucidated the target genes directly bound by these proteins and characterized important transcriptional regulators involved in the hematopoietic program (93). In particular, researchers uncovered the genome-wide binding sites of ten crucial transcription regulators (SCL, LYL1, LMO2, GATA2, RUNX1, MEIS1, PU.1, ERG, FLI-1, and GFI1B) in a hematopoietic progenitor cell line HPC-7 and proposed the interaction of a heptamer complex consisting of SCL/ LYL1/ GATA2/ RUNX1/ LMO2/ FLI-1/ ERG in hematopoietic regulation (93). Select transcription factors that are crucial for the emergence of HSCs will be discussed in more detail below.

Hematopoietic lineage specification and differentiation are also governed by transcription factors, such as PU.1, GATA1, IKAROS, and GATA3 (3, 6, 94). PU.1 plays a role in differentiation of the myeloid lineage (3). GATA1 functions in differentiation of the erythroid and megakaryocytic lineage (3). IKAROS is required for differentiation of the lymphoid lineage (6). GATA3 participates in the differentiation of T-cell lineage (94). The transcription factors involved in early and/ or late B-cell development include E2A, EBF1, PAX5, IRF4, IRF8, BCL6, BLIMP1, XBP1, and more (6, 95). These select transcription factors in B-cell lymphopoiesis will be discussed in more detail below.

1.3.2.1 SCL/ TAL1

Stem cell leukemia/ T-cell acute lymphoblastic leukemia [T-ALL] 1 (SCL/ TAL1) is part of the basic helix-loop-helix (bHLH) family of transcription factors and is originally identified due to its frequent overexpression in T-ALL as a result of chromosomal translocations (96). Mouse embryos with SCL-deficiency showed a lack of hematopoiesis within the yolk sac and died on around embryonic day 9.5 (E9.5) (97, 98). *Scl*-deficient embryonic stem cells were also unable to contribute to primitive or definitive hematopoiesis in chimeric mice, indicating the requirement of SCL in the development of all lineages in the hematopoietic system (99, 100).

1.3.2.2 LMO2

Lin11/ Isl1/ Mec3 (LIM) domain only 2 (LMO2) is a protein that is aberrantly expressed in T-cell leukemia through mechanisms of chromosomal translocation (101, 102). Mouse embryos with LMO2-deficiency showed a lack of erythropoiesis within the yolk sac and died on around E10.5 (103). In the erythroid lineage, LMO2 was shown to interact with SCL, E2A, GATA-1, and LDB1 to form a DNA-binding transcriptional transactivating complex (104). *Lmo2*-deficient embryonic stem cells were also unable to give rise to any adult blood cells in chimeric mice, demonstrating the importance of LMO2 in the emergence of hematopoiesis (105).

1.3.2.3 GATA2

GATA-binding factor 2 (GATA2) is part of the zinc finger family of transcription factors (106). Mouse embryos deficient for GATA2 showed decreased primitive hematopoiesis and pronounced anemia, with most embryos dead around E10-11 (106). In chimeric mice, *Gata2*-deficient embryonic stem cells were impaired in the contribution towards primitive hematopoiesis and were

completely unable to participate in definitive hematopoiesis (106). These findings illustrate the importance of GATA2 in hematopoietic development (106).

1.3.2.4 RUNX1

Runt-related transcription factor 1 (RUNX1), also known as acute myeloid leukemia 1 (AML1) or core-binding factor subunit alpha-2 (CBFA2), is part of the Runt-related family of transcription factors and complexes with CBFβ to form CBF (107). Chromosomal translocations of genes encoding these subunits are commonly found in leukemia and myelodysplasia (107). Mouse embryos with RUNX1-deficiency died around E12.5, with presence of hemorrhages within the central nervous system (108). Although *Runx1*-deficient embryos had primitive erythropoiesis in the yolk sac, they lacked definitive hematopoiesis in the fetal liver (108). *Runx1*-deficient embryonic stem cells were also unable to participate in definitive hematopoiesis in chimeric mice, demonstrating the significance of RUNX1 in forming definitive hematopoiesis (108).

1.3.2.5 PU.1

PU.1 is part of the ETS (Erythroblast Transformation Specific) family of transcription factors and is needed for HSC self-renewal, HSC differentiation into CMPs and CLPs, along with granulocyte and monocyte maturation (109). PU.1 is highly expressed in myeloid cells, but relatively lowly expressed in B cells (110). In early lymphoid progenitors, PU.1 regulates the expression of two cytokine receptors (FLT3 and IL-7Rα) (111, 112). PU.1 is needed for the generation of lymphoid progenitor cells but is not required for the maturation of B cells; PU.1-deficient mice lack CLPs, while conditional knockout mice with PU.1-deficient mature B cells show normal B-cell maturation and proliferation (109).

1.3.2.6 E2A

E2A is part of the helix-loop-helix family of transcription factors (113). In HSCs and LMPPs, E2A is essential for priming the expression of lymphoid lineage genes while repressing the expression of HSC-genes and non-lymphoid genes (113). E2A is also important for regulating many genes involved in the B cell program, commonly together with transcription factors EBF1 and PAX5 (114, 115). E2A-deficient mice show reduced LMPPs and CLPs and a block at the pre-pro-B cell stage, resulting in the absence of mature B cells (113, 116, 117). E2A-deficient progenitor cells do not activate the transcription of *Ebf1* and *Pax5*, as well as *Rag1*, so they cannot perform immunoglobulin gene recombination (116, 118).

1.3.2.7 EBF1

Early B-cell Factor 1 (EBF1) is part of the helix-loop-helix family of transcription factors and functions together with E2A to activate transcription of B cell genes (114, 115). EBF1 upregulates several genes essential for B-cell development (such as *Igα*, *VpreB*, *λ5*, and *Pax5*) and silences genes of other lineage fates (such as *Gata3* for T-cell differentiation) through binding to the gene promoter and recruiting repressive modifications (115, 119-122). EBF1-deficient mice are arrested at the pre-pro-B cell stage of development, unable to progress to pro-B cells, likely as a result of a lack of B-cell associated genes (123).

1.3.2.8 PAX5

PAX5 is part of the paired-box (PAX) family of transcription factors and is induced by E2A and EBF1 to confine progenitors to the B-cell lineage (124). PAX5 directly regulates hundreds of genes in B cells, many of which are necessary for B-cell function (95). PAX5 upregulates the expression

of the B cell lineage genes (e.g. *Cd19* and *Igα*) and genes encoding transcriptional regulators needed in pre-B cells (e.g. *Aiolos*, *Bach2*, *Irf4*, and *Irf8*), while downregulating the expression of genes involved in multipotent progenitor signalling (e.g. *Flt3*) and genes of non-B cell lineages (e.g. *Notch1* for T-cell development and the myeloid *Mcsfr*) (124-129). As a result, PAX5-deficient mice are blocked at early pro-B cells, with PAX5-deficient pro-B cells able to differentiate into other blood cells, but not mature B cells (124, 127).

1.3.2.9 IRF4 and IRF8

Interferon Regulatory Factor (IRF) 4 is a member of the IRF family of transcription factors and plays important roles in B cells (95). In pre-B cells, IRF4 (i) attenuates IL-7R signalling by upregulating CXCR4 expression and promoting chemotaxis towards CXCL12-expressing stromal cells and (ii) supports light chain recombination through enhancing histone acetylation at immunoglobulin light chain enhancer regions (130). In immature B cells, upon the encounter of self-antigen, enhanced IRF4 expression is required to induce secondary rearrangement of the immunoglobulin light chain (receptor editing), helping to maintain B-cell tolerance (131). Although IRF4-deficient mice have normal surface markers of early progenitor B cells and immature B cells, they show impaired maturation in the peripheral lymphoid organs (132). In peripheral B cells, IRF4 is needed for germinal centre B cell formation, class switch recombination, and plasma cell differentiation (133, 134). In peripheral B cells, IRF4 is required at a low and transient level to drive the expression of *Aicda* (encoding AID) and *Bcl6* for GC B cell formation (134). Then later on, a high and maintained level of IRF4 is needed to reduce the expression of *Bcl6* while upregulating *Blimp1*, which facilitates exit from the GC program and orchestrates plasma cell differentiation, respectively (134).

IRF8 is also a member of the IRF family of transcription factors, with high homology to IRF4 (135). IRF8-deficient mice have decreased CLPs and greatly reduced pre-pro-B cells; this phenotype is linked to a downregulation of genes encoding B-cell transcription factors E2A, EBF1, and PAX5 (136). *Irf4*- and *Irf8*-double knockout mice are blocked at the pre-B cell stage, with hyperproliferative pre-B cells greatly expressing surface pre-BCR and lacking immunoglobulin light chain rearrangement (135). IRF4 or IRF8 are recruited by transcription factor PU.1 to their binding sites within immunoglobulin Igκ and Igλ enhancer regions and function together as a transcriptional activator (137, 138). IRF4 and IRF8 also promote pre-B cell transition together by activating the expression of IKAROS and AIOLOS, which downregulate surrogate light chain and pre-BCR expression and suppress pre-B cell proliferation (139).

1.3.2.10 BCL6

B-cell lymphoma 6 (BCL6) is part of the zinc finger family of transcription factors and is critical for the formation of GC B cells (140). Upon engaging with their antigen in the early phase of the GC response, B cells interact with follicular helper T cells (T_{FH}) and upregulate the expression of *Bcl6* (140). BCL6 in turn maintains the interaction between T_{FH} and B cells in a positive feedback loop and allows cells to enter GC clusters, driving the GC program (140). As a transcriptional repressor, BCL6 also suppresses the expression of p53 in GC B cells, protecting cells from DNA damage-induced apoptosis (141).

1.3.2.11 BLIMP1 and XBP1

B-lymphocyte-induced maturation protein 1 (BLIMP1) is part of the zinc finger family of transcription factors and triggers the differentiation of GC B cells into plasma cells (142, 143). As a transcriptional repressor, BLIMP1 silences the expression of *Pax5*, removing the PAX5-mediated activation of *Aicda* and *Bcl6* and repression of *Xbp1* (142-144). X-box binding protein 1 (XBP1) is a basic leucine zipper (bZIP) transcription factor that expands the endoplasmic reticulum and induces genes encoding the protein secretory pathway, collectively promoting the increased production of immunoglobulin molecules (145). Therefore, in addition to suppressing the GC program, BLIMP1 contributes to the mass secretion of antibodies by plasma cells with the derepression of XBP1 (143).

1.3.3 Epigenetic Regulation

Within the cell nucleus, DNA wraps around histone octamers into units of nucleosomes that fold up to form chromatin fibres, compacting a great amount of genetic information in a condensed state (146, 147). During cellular development, distinct sets of genes are expressed or repressed by different cell types, and this process of gene transcription is regulated through epigenetic modifications, which are changes to the chromatin besides altering the DNA sequence (30). Epigenetic modifications include methylation of DNA at sites of CpG dinucleotides (associated with gene silencing), post-translational modifications on histones, and regulation of these processes by non-coding RNAs (148, 149). Patterns of epigenetic modifications are generated during development and persist with cell division (30). Aberrant alterations in these epigenetic modifications may lead to dysregulation of tumour suppressor genes and/ or oncogenes, thereby contributing to malignant transformation (30).

1.3.3.1 Histone Modifications

The histone octamer of a nucleosome is composed of two of each of the core histones (H2A, H2B, H3, H4), connected by an H1 linker (150). The core histones extend tails outwards, serving as sites for decoration by various modifications, such as acetylation, methylation, phosphorylation, sumoylation, and ubiquitination (150, 151). These histone marks are added by "writer" enzymes, recognized by "reader" proteins, and removed by "eraser" enzymes (150). Alterations of the levels of histone modifications or the levels of writers, readers, and erasers have been associated with cancer (30). Histone modifications may affect the interactions between positively charged amino acid residues of histones and negatively charged DNA phosphate backbone, and they may also recruit "readers" like architectural proteins (which bind to several nucleosomes at once and block

the access to DNA or tighten chromatin), chromatin remodeling complexes (which move nucleosomes and allow access to DNA), secondary chromatin modifiers (which act on the current modification or make additional changes to the chromatin), or adaptors (which recruit proteins involved in transcription, DNA damage repair, and other DNA metabolism processes) (6, 30, 146, 147, 150). Altogether, these modifications alter chromatin structure and modulate the accessibility of DNA, facilitating vital biological processes during development (30, 146).

1.3.3.2 Epigenetic Regulation of Hematopoiesis

Hematopoietic proliferation and differentiation are also governed by multiple chromatin-modifying enzymes, including histone acetyltransferases/ deacetylases, histone methyltransferases/ demethylases, and DNA methyltransferases (30). (i) Histone acetylation by MOZ is required for proliferation of HSCs and development of B cells, whereas histone acetylation by HBO1 promotes the differentiation of erythroid progenitors (152-154). Removal of histone acetylation by HDAC1 is involved in erythroid and megakaryocytic differentiation (155). (ii) Methylation of histone H3-K27 by EZH2 and of H3-K4 by MLL are important for fetal liver hematopoiesis (156-158). Methylation of H3-K36 by ASH1 plays a role in myeloid differentiation, whereas methylation of H3-K79 by DOT1L participates in erythroid progenitor differentiation (159, 160). Removal of methylation on H3-K4 and H3-K9 by LSD1 is involved in restraining proliferation of hematopoietic progenitors and in differentiation of the erythroid lineage (161). (iii) DNA methylation by DNMT3a and DNMT3b regulate self-renewal and differentiation of HSCs (162, 163). The expression or function of these chromatin-modifying enzymes is often dysregulated in hematologic malignancies, illustrating the crucial roles of histone- and DNA-modifying enzymes in epigenetic regulation during normal hematopoietic development (30).

1.4 Introduction to Histone Ubiquitination and Deubiquitination

1.4.1 Ubiquitination and Deubiquitination

In addition to the modifications mentioned above, histones may also be decorated with ubiquitin. Ubiquitin is a 76-amino acid protein that is appended to target proteins via an isopeptide bond between the C-terminal carboxyl group of a glycine residue of ubiquitin and the ε-amino group of a lysine residue on the protein substrate (164). This process of ubiquitination is catalyzed by three enzyme families: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) (164). E1 first covalently links via its cysteine residue to a ubiquitin molecule in an ATP-dependent manner (164). Then, E1 transfers this activated ubiquitin to E2 (164). While carrying the ubiquitin molecule, E2 binds E3, which recognizes and binds the target protein (164). Finally, E3 attaches the ubiquitin from E2 onto the protein substrate (164). Ubiquitin itself contains seven lysine residues, allowing the linkage between ubiquitin molecules and formation of polyubiquitin chains on target proteins (165). Monoubiquitination and K63-linked polyubiquitination (composed of ubiquitin molecules connected through the lysine residue at position 63 of ubiquitin) often alters localization of the protein or modifies its interaction with other proteins or DNA, whereas K48-linked polyubiquitination usually targets the protein for degradation by the proteasome (165). E3 ubiquitin ligases are categorized into three classes: Really Interesting New Gene (RING), Homologous to E6AP C-Terminus (HECT), and RING-between-RING (RBR) (164).

On the other hand, deubiquitinating enzymes, or deubiquitinases (DUBs), catalyze the removal of ubiquitin, modulating the levels of protein ubiquitination (166). DUBs are grouped into seven families: ubiquitin-specific protease (USP), ovarian tumour (OTU), JAB1/ Mpr1 Pad1 N-

terminal/ Mov34 metalloenzyme (JAMM), Machado-Joseph disease protein domain (MJD), ubiquitin C-terminal hydrolase (UCH), motif interacting with ubiquitin-containing novel DUB family (MINDY), and zinc finger containing ubiquitin peptidase 1 (ZUP1) (166). The latter two families of DUBs were discovered relatively recently (167, 168).

1.4.2 Histone (De)ubiquitination in Cellular Processes

Even though histone H2A was the first ubiquitinated protein identified, ubiquitination remains one of the least well-characterized histone modifications (169, 170). All the core histones, as well as the H1 linker, may be subjected to ubiquitination; however, the specific ubiquitination sites and their associated biological functions are more well-known for histones H2A and H2B (171-173). In vertebrate cells, approximately 10% of H2A and 1% of H2B are monoubiquitinated (170, 174, 175). The steady state of ubiquitinated histones is modulated by histone ubiquitin ligases and counteracting histone DUBs. Histone ubiquitin ligases are mainly from the RING family (e.g., those discussed in the following sections—RING1B, RNF8, RNF168, and BRCA1 for H2A, along with RNF20 and RNF40 for H2B). Histone DUBs are mainly from four DUB families, specifically the USP, JAMM, UCH, and OTU families. Histone (de)ubiquitination plays an important role in the regulation of cellular processes, including gene transcription, DNA damage repair, and cell cycle progression (16, 170, 173, 176-180). As aberrations in the levels of ubiquitinated histones and the associated writers, readers, and erasers are implicated in cancer, studies elucidating the functions of histone (de)ubiquitination are beneficial and valuable (170, 173).

1.4.2.1 Gene Transcription

Ubiquitination of histones functions to regulate gene transcription. The predominant form of ubiquitinated H2A is monoubiquitinated histone H2A at lysine 119 (H2A-K119ub, or uH2A for short), which is mainly catalyzed by RING1A/ RING1B/ B-lymphoma Moloney murine leukemia virus insertion region 1 homolog (BMI1) subunit of the Polycomb repressive complex 1 (PRC1), composed of proteins that cooperatively silence gene expression partially through interacting with the chromatin (16, 173). H2A-K119ub is found in satellite DNA regions and often associated with transcriptional repression (e.g., Homeobox (HOX) gene silencing and X-chromosome inactivation) (16, 170, 179, 181-184). Some of the functions of H2A-K119ub include: (i) blocking the recruitment of FACT (facilitates chromatin transcription) protein complex to gene promoters, ultimately preventing transcription elongation, (ii) inhibiting the formation of gene-activating H3-K4 di- and tri-methylation marks, resulting in repression of transcription initiation, and (iii) recruiting the PRC2 that forms the gene-silencing H3-K27 trimethylation mark (173, 185-188). Removal of H2A-K119ub also functions in gene regulation and may be catalyzed by various H2A DUBs, such as BRCA1-associated protein-1 (BAP1), Myb-like SWIRM and MPN domains 1 (MYSM1), ubiquitin-specific protease 16 (USP16), and USP21 (27, 176, 186, 189, 190).

The predominant form of ubiquitinated H2B is monoubiquitinated histone H2B at lysine 120 (H2B-K120ub, or uH2B for short), which is primarily catalyzed by the ring finger protein 20 (RNF20)/ RNF40 E3 ubiquitin ligase complex (173, 175, 191). H2B-K120ub has been associated with both transcriptional activation and repression (192). Mechanisms of H2B-K120ub in gene activation include: (i) impeding the compaction of chromatin, (ii) assisting transcription elongation by RNA polymerase II together with FACT protein complex, and (iii) enhancing the formation of gene-activating marks H3-K4 di- and tri-methylation, mediated by the SET1 complex, and H3-

K79 methylation, mediated by disruptor of telomeric silencing 1-like (DOT1L) methyltransferase (173, 193-196). Removal of H2B-K120ub may be catalyzed by H2B DUBs, such as USP22, USP44, USP49 (27, 197-199). Through regulating gene expression, histone (de)ubiquitination plays a role in the maintenance and differentiation of stem cells, as well as other cellular functions, such as proliferation, migration, and cell death (200-203).

1.4.2.2 DNA Repair

Histone ubiquitination participates in DNA damage repair. The ubiquitination sites on histones H2A/ variant H2AX that are involved in the DNA damage response include: K15, K119, K127, and K129 (173). In response to DNA double-strand break, nearby H2AX quickly becomes phosphorylated on the serine residue at position 139 (known as γH2AX) by protein kinases ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3-related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs), promoting the association of regulatory proteins (177, 204, 205). Among those are histone ubiquitin ligases RNF8/ RNF168 that catalyze the monoubiquitination and K63-linked polyubiquitination of H2A-K15, which then recruit tumor suppressor p53-binding protein 1 (53BP1) and the breast cancer type 1 susceptibility protein-associated (BRCA1-A) complex, respectively, to help regulate DNA end resection (172, 173, 177, 206-213). BRCA1 near the DNA damage site also catalyzes the addition of H2A-K127ub and/ or H2A-K129ub, which recruits chromatin remodeling protein SMARCAD1 (or SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily a, containing DEAD/H box 1) to enhance DNA end resection (173, 213-215). Furthermore, following DNA double-strand break, deposition of H2A-K119ub, dependent on BMI1 of the PRC1, silences transcription of flanking genes, contributing to effective DNA repair (216, 217). Besides histone H2A

ubiquitination, addition of H2B-K120ub, catalyzed by RNF20/ RNF40, recruits more DNA repair factors involved in homologous recombination (HR) and non-homologous end joining (NHEJ) to the sites of DNA damage (218, 219). And so, the processes of H2A and H2B ubiquitination collectively participate in efficient DNA damage repair.

Interestingly, reversal of histone ubiquitination also plays essential roles in the DNA damage response. Histone DUB BAP1 removes H2A-K119ub and associates with the recruitment of HR repair proteins to regions of DNA damage (220). USP48 opposes BRCA1-mediated H2A ubiquitination and functions to restrict DNA resection (221). Further from the DNA damage site, BRCA1/ BRCA2-containing complex subunit 36 (BRCC36) counteracts RNF8-dependent K63-linked polyubiquitination events, restrains DNA end resection, and represses HR repair (213, 222, 223). Removal of H2B-K120ub by USP22 is also needed for HR, NHEJ, V(D)J recombination, and class-switch recombination (224, 225). After DNA repair, USP51 counteracts H2A-K15ub and promotes 53BP1 disassembly (226). Together, previous studies suggest that dynamic histone ubiquitination and deubiquitination cooperate to prevent chromatin compaction and enhance recruitment of regulatory proteins to sites of DNA damage for effective DNA repair (193, 219).

1.4.2.3 Cell Cycle Progression

Deubiquitination of histones associates with cell cycle progression. Histone DUBs may directly participate in cell cycle control by removing the potentially obstructive ubiquitin mark (179). Normally, uH2A levels were reduced upon entering the mitotic (M) phase of cell cycle and were reestablished following exit of the M phase, up towards the G1/S phases (179). As ubiquitinated histones were proposed to disrupt the binding of Aurora B kinase to nucleosomes, removal of uH2A enabled Aurora B kinase to perform phosphorylation of serine 10 on histone H3 (H3S10p),

a subsequent step in the cell cycle process (179). Consistently, uH2A levels inversely correlated with H3S10p levels during cell cycle (179). Furthermore, depletion of H2A-DUB USP16 delayed the M phase and slowed cell proliferation, supporting that H2A deubiquitination catalyzed by USP16 is necessary for cell cycle M phase progression (179).

Histone DUBs may also have indirect effects on the cell cycle through their roles in DNA damage response and/ or gene transcription or through the functions of their non-histone protein substrates. The first example of a histone DUB indirectly affecting cell cycle is USP3, through maintaining DNA stability (227). Deficiency of USP3 slowed down cell cycle S phase progression and mitotic entry, with USP3-knockdown cells having fewer mitotic (phosphorylated histone H3-positive) cells (227). In addition, loss of USP3 associated with the presence of single-stranded DNA and elevated levels of DNA damage response markers, such as γ-H2AX, 53BP1, and activated targets of ATM and ATR kinases (227). Hours after ionizing radiation, USP3-deficient cells also retained upregulated γ-H2AX and uH2A nuclear foci, unable to effectively dissipate them (227). These data suggested that cell cycle defects in USP3-knockdown cells might have resulted from prolonged histone ubiquitination, unresolved DNA damage, and activation of cell cycle checkpoint (227). And so, regulation of H2A deubiquitination by USP3 is needed for cell cycle S phase progression through the preservation of genome integrity (227).

The second example of a histone DUB indirectly acting on the cell cycle is USP36, through the regulation of gene transcription (201). Depletion of USP36 enhanced uH2B and H3K4me3 marks at the gene locus for cyclin-dependent kinase inhibitor 1A (*Cdkn1a*), which encodes the cell cycle inhibitor p21, promoting *Cdkn1a* expression and repressing cell proliferation (201). These findings demonstrated that H2B deubiquitination by USP36 modulated cell proliferation via influencing expression of a gene involved in the cell cycle (201). The third example of a histone

DUB indirectly participating in the cell cycle is USP22, through the downstream function of its non-histone target (228). By removing K63-linked polyubiquitin chain on far upstream element (FUSE)-binding protein 1 (FUBP1), USP22 promoted the binding of FUBP1 and FUBP1-interacting repressor (FIR) to the FUSE region of the *Cdkn1a* gene, silencing the expression of p21 (228). Consistently, knockdown of USP22 resulted in impaired cell proliferation mediated by p21, with depletion of p21 in USP22-knockdown cells restoring the cell growth phenotype (228). These results showed that USP22 indirectly played a role in the cell cycle through deubiquitination of a non-histone substrate (228). Altogether, multiple histone DUBs are documented to control the cell cycle by protein deubiquitination.

1.4.3 Histone Deubiquitination in Hematologic and Immune Systems

The functions of histone deubiquitinases (DUBs) in mammalian physiology have been elucidated rather recently (229, 230). Several histone DUBs participate in the regulation of hematopoietic and immune systems (225, 229-234). For a number of these histone DUBs, their catalytic activity on histone substrate contributes to their role in hematopoiesis (e.g., USP3, USP16, and USP22, summarized in the table below, while BAP1 and MYSM1 are reviewed in subsequent subsections) (225, 231-233). For some histone DUBs, their significance in hematopoietic and immune systems is due to their deubiquitinating action on non-histone substrates, affecting the stability and/ or function of target proteins (235-237). Given the variety of histone DUBs and DUB functions, a couple of histone DUBs have also been shown to be dispensable in certain aspects of the blood system, likely because of functional redundancy (e.g., USP22 in myelopoiesis, as well as USP21 and USP44 in hematopoietic stem cell function and lymphopoiesis) (2, 238, 239). Future studies are needed to fully characterize the catalytic involvement of the remaining histone DUBs to

determine the essential histone DUBs, for a better understanding of the importance of this epigenetic modification in the blood and immune systems.

Histone DUB	Family	Histone Substrate(s)	Function in Hematopoiesis	References
USP3	USP	H2A(X), H2B	Maintains HSC self-renewal and DNA damage response, protects HSCs and mice against genotoxic stress, removes uH2A and uH2B in adult tissues	(231)
USP16	USP	H2A	Promotes HSC cell cycle entry and cell differentiation, represses the expression of *Cdkn1a* encoding p21cip1, counteracts PRC1-mediated uH2A in the transcriptional regulation of HSPCs	(232)
USP22	USP	H2A, H2B	Promotes transition of iNKT cells during development, interacts with MED1 to activate essential iNKT cell genes, removes uH2A at the promoters of *IL-2Rβ* and *T-bet* in iNKT cells Facilitates c-NHEJ DNA repair in B cells (including V(D)J recombination and class-switch recombination to IgG/IgE antibody isotypes), removes uH2B in splenic B cells	(225, 233)

Table 1.4.1 Examples of Hematopoietic Functions Performed by Histone Deubiquitinases.

Abbreviations. USP: ubiquitin-specific peptidase; HSC: hematopoietic stem cells; *Cdkn1a*: cyclin-dependent kinase inhibitor 1A; PRC1: Polycomb repressive complex 1; HSPC: hematopoietic stem and progenitor cells; iNKT: invariant natural killer T; MED1: mediator of RNA polymerase II transcription subunit 1; *IL-2Rβ*: IL-2 receptor β chain; *T-bet*: T-box transcription factor; c-NHEJ: classical non-homologous end joining

1.5 BRCA1-associated protein 1 (BAP1)

1.5.1 Discovery of BAP1

BAP1 is a deubiquitinase of the Ubiquitin C-terminal Hydrolase (UCH) family (240). BAP1 is involved in many cellular processes, such as transcriptional regulation, DNA repair, and tumour suppression (189, 220, 234, 241, 242). These functions of BAP1 are mediated through BAP1-catalyzed deubiquitination of histone H2A-K119ub and through BAP1 effects on the ubiquitination, stability, and localization of other transcriptional regulators (240, 241, 243). Through a yeast-two hybrid screen, BAP1 was initially introduced as a binding partner of the RING finger domain of Breast Cancer 1, Early Onset (BRCA1), a protein essential for DNA double-strand break repair; however, further experiments using mass spectrometry did not detect BRCA1 in BAP1 protein complex (241, 244). Later studies also concluded that BAP1 does not depend on BRCA1 for its tumour suppression, conflicting with the initially proposed mechanism of BRCA1-involvement in BAP1 function (240, 242).

1.5.2 BAP1 Protein Structure and Complexes

The protein structure of BAP1 consists of the UCH enzymatic domain at its N-terminus, a functional nuclear localization sequence (NLS) at its C-terminus, plus several protein-interacting domains in the middle, including BRCA1-associated RING domain 1 (BARD1)-binding domain, host cell factor 1 (HCF1)-binding motif, Yin Yang 1 (YY1)-binding region, and additional sex combs like 1 and 2 (ASXL1/2)-binding regions (189, 240, 245). The UCH enzymatic domain allows BAP1 to deubiquitinate histone H2A-K119ub and preferentially hydrolyze K48-linked over K63-linked polyubiquitin chains (220, 243). The NLS brings BAP1 into the nucleus, where it

mainly resides (246). The localization of BAP1 is regulated by opposing activities: (a) ubiquitin-conjugating enzyme E2 O (UBE2O)-mediated ubiquitination on the NLS of BAP1, which sequesters BAP1 in the cytoplasm, and (b) the counteractive BAP1-mediated auto-deubiquitination (246). Protein-binding domains enable BAP1 to participate in large multi-protein complexes and to coordinate with other proteins in regulating cellular processes; some of the BAP1-associated complexes are composed of a combination of: HCF1, YY1, ASXL1/2, O-linked β-N-Acetylglucosamine (O-GlcNAc) Transferase (OGT), and Forkhead Box Protein K1 and K2 (FOXK1/2) (189, 240, 241).

1.5.3 Transcriptional Regulation by BAP1 Protein Complexes

Within the nucleus, BAP1 regulates expression of genes involved in metabolism, cell proliferation, cell death, and more processes, by participating in multi-protein complexes (189, 203, 241, 242, 246, 247). Transcriptional regulation by BAP1 correlates with BAP1-dependent deubiquitination (203).

1.5.3.1 Cell proliferation

In the BAP1-HCF1 complex, BAP1 deubiquitinates HCF1, enabling HCF1 to recruit histone methyltransferases to the E2F transcription factor 1 (E2F1) that then activates transcription of E2F1-target genes (240, 248). Therefore, the BAP1-HCF1 complex is indirectly involved in cell cycle regulation and cell proliferation (248). In the BAP1-ASXL1/2 complex, also known as the Polycomb repressive deubiquitinase complex (PR-DUB), BAP1 also has a similar role. BAP1-deficient cells showed a defect in cell progression from the G1 to S phase of the cell cycle (189, 248). This cell proliferation defect was rescued when wild type BAP1 was introduced back into

BAP1-deficient cells, but not when BAP1 construct lacking the catalytic domain or the ASXL1/2 interaction domain was introduced (189). And so, the BAP1-ASXL1/2 complex, together with BAP1 catalytic function, is indispensable for the role of BAP1 in cell proliferation (189). By controlling the expression of multiple genes, BAP1 affects a wide range of cellular functions (240). This function of BAP1 appears to depend on the associated factor(s) present in its protein complex, suggesting that BAP1 may have cell type-specific and temporally defined roles (240). As such, we are interested in the potential role of BAP1 in regulating different stages of B-cell development.

1.5.3.2 Metabolism

In the BAP1-HCF1-OGT complex, BAP1 deubiquitinates and stabilizes OGT, which attaches O-GlcNAc on and activates HCF1 transcriptional regulator (240, 249). In turn, HCF1 recruits OGT to add O-GlcNAc to the peroxisome proliferator activator receptor γ coactivator 1α (PGC1α), allowing BAP1 to interact, deubiquitinate, and stabilize PGC1α (249). Therefore, the BAP1-HCF1-OGT complex is associated with regulation of gluconeogenesis and mitochondrial biogenesis (240, 249). In the BAP1-HCF1-YY1 complex, BAP1 and HCF1 are recruited by YY1, a transcription factor involved in cell proliferation, to the promoter region of *Cox7c* gene, which encodes a component of mitochondrial electron transport chain (241). BAP1 assembles HCF1 and YY1 through its protein-interacting domains, enabling the transcriptional activation of the *Cox7c* gene (241). And so, the BAP1-HCF1-YY1 complex regulates mitochondrial function and metabolism (241).

1.5.4 BAP1 in Tumour Suppression and the Hematologic System

As BAP1 mutations are linked to many types of human malignancies, BAP1 is a highly important tumour suppressor (240). In humans, BAP1 deletions or mutations are commonly found in metastasizing uveal melanoma (84%), malignant pleural mesothelioma (47-60%), and renal cell carcinoma (15%) (250-253). In mice, the tumour suppressor activity of BAP1 is conserved. *Bap1*-knockout mice are embryonic lethal, while *Bap1*-heterozygous mice have a greater tendency than wild type mice to develop spontaneous tumours (234, 254). At the molecular level within these BAP1-cancers, BAP1 is frequently mutated in the nuclear localization sequence, the ASXL1/2 interaction domain, and the catalytic domain, indicating the importance of BAP1 retention in the nucleus, BAP1/ASXL interaction, and ASXL1/2-mediated activation of BAP1-deubiquitinase activity in tumour suppression (242). Consistent with the molecular data highlighting the importance of BAP1/ASXL axis in tumour suppression, inducible systemic deletion of *Bap1* in *Bap1*$^{fl/fl}$*Cre*^{ERT2} mice showed a phenotype that resembled myelodysplastic syndrome (MDS) and chronic myelomonocytic leukemia (CMML), which are blood cancers frequently associated with ASXL1 mutations and characterized by an expansion of myeloid cells, the presence of immature precursor blood cells, and a reduction of red blood cells and platelets (234). “These mice also exhibited impaired thymocyte differentiation and diminished peripheral T cell expansion, with disruptions in the transcriptional programs of cell cycle progression in T cells (255)” (1). The involvement of BAP1 in T-cell development and proliferation was mediated by its deubiquitinase activity (255). “Importantly, depletion of B cells was also observed following the systemic inducible deletion of *Bap1* in *Bap1*$^{fl/fl}$*Cre*^{ERT2} mice (255). However, this experimental model did not allow characterization of the cell-intrinsic functions of BAP1 in the B cell lineage, independently of BAP1 functions in the precursor hematopoietic stem and progenitor cells (234),

and independently of other cells that interact with B cells to regulate their maturation and engagement in immune response (255). Furthermore, BAP1 molecular functions and mechanisms of action in the B cell lineage were not explored in the previous studies" (1). Overall, these results emphasize the significance of BAP1 in maintaining hematopoietic lineages and merit further investigation of the poorly understood functions of BAP1 in B cells.

1.6 Myb-like, SWIRM and MPN Domains 1 (MYSM1)

1.6.1 MYSM1 in Hematopoiesis

Similar to BAP1, MYSM1 is a deubiquitinase important for maintaining hematopoiesis and lymphopoiesis (230). *Mysm1*-knockout mice showed developmental defects, abnormal hind limb and tail development, and severe hematopoietic dysfunction (230). Deletion of *Mysm1* resulted in loss-of-quiescence in hematopoietic stem cells (HSCs), impaired HSC self-renewal and differentiation, increased apoptosis in hematopoietic progenitors, as well as reduced mature progenitor cells and downstream blood cells (230, 256). Strikingly, within the affected cell populations, B cells were the most depleted in the bone marrow of *Mysm1*-knockout mice (230). In humans, deficiency of MYSM1 or presence of only the catalytically inactive version of MYSM1 also results in anemia, lymphopoietic deficiencies, and bone marrow failure, demonstrating that the regulation of hematopoiesis by MYSM1 is conserved in mice and humans (257-259).

1.6.2 MYSM1 Protein Structure

The protein structure of MYSM1 is composed of the Myb-like SANT domain, which allows interaction with DNA and histone tails; the SWIRM domain, which enables interaction with histone H3; and the MPN+ domain containing a JAMM motif, which carries the catalytic activity (176, 260). In particular, MYSM1 is a deubiquitinase from the metalloprotease family, which binds to zinc ions and catalyzes the attack of a water molecule on an isopeptide bond between amino acids of proteins, such as the bond linking a protein with ubiquitin (257). Together, these domains allow MYSM1 to associate with chromatin and hydrolyze isopeptide bonds to remove ubiquitin molecules, mainly from histone H2A-K119ub (257).

1.6.3 Histone Orchestration by MYSM1

One of the proposed mechanisms of action of MYSM1 places this deubiquitinase in a complex with histone acetyltransferase pCAF at histone H2A-K119ub regions (176). In a prostate cancer cell line, pCAF-MYSM1 is recruited to target gene promoters, where pCAF catalyzes the addition of an acetyl group, and MYSM1 catalyzes the removal of H2A-K119 monoubiquitin from acetylated nucleosomes, enabling the release of linker histone H1 from nucleosomes (176). These chromatin modifications in turn produce a conformation that favours the recruitment of initiating Pol II complex to the promoters for gene transcription (176). And so, pCAF-MYSM1 participates in transcriptional regulation through sequential operation of histone acetylation, histone deubiquitination, and linker histone release (176). In hematopoietic cells, MYSM1 promotes gene expression by deubiquitinating H2A-K119ub at the *Gfi1* locus, for maintenance of HSC homeostasis; the *Flt3* locus, for dendritic cell development; the *Id2* locus, for natural killer cell development; and the *Ebf1* locus, for B-cell commitment and development (229, 261-263). Histone deubiquitination at those regions also promotes addition of permissive histone modification, induces removal of repressive histone modifications, and recruits important transcription factors, such as GATA2 and RUNX1 to the *Gfi1* locus, PU.1 to the *Flt3* locus, NFIL3 to the *Id2* locus, and E2A to the *Ebf1* locus (229, 261-263). Collectively, these studies associate histone deubiquitination by MYSM1 with gene de-repression, or gene activation.

1.6.4 The MYSM1/ p53 Axis in Hematopoiesis

As a consequence of MYSM1-deficiency, the p53 stress-response pathway is induced (264, 265). Hematopoietic cells from *Mysm1*-deficient mice showed increased p53 protein abundance, higher expression of p53 stress-response target genes, and enhanced p53-dependent apoptosis (264-267).

Deletion of p53 was able to rescue the developmental and hematologic phenotypes of *Mysm1*-knockout mice, supporting p53 as a mediator and driver of the abnormalities observed in MYSM1-deficient mice (265, 268). More specifically, *Mysm1*$^{-/-}$*p53*$^{-/-}$ (double-knockout) mice are normal in appearance and have restored HSC function, with normal cell count and development of HSCs, multipotent progenitors, lymphoid and myeloid progenitors, B cells, and T cells (265, 268). At the molecular level, MYSM1 and p53 are able to separately bind to promoters of p53-target genes (encoding PUMA and p21), suggesting a cooperation between MYSM1 and p53 in transcriptional regulation (264). In addition, MYSM1 also influences histone acetylation and methylation at these p53-target gene loci (264). However, the exact mechanism leading to p53 activation in *Mysm1*-knockout mice requires further investigation.

Through integrating ChIP-seq and RNA-seq data, our lab has recently demonstrated MYSM1 as a regulator of ribosomal protein (*RP*) genes in hematopoietic stem and progenitor cells (269). In addition to reduced *RP*-gene expression, hematopoietic stem cells from *Mysm1*-deficient mice also showed decreased protein synthesis rate and elevated p53 protein level, suggesting that dysregulation in *RP*-gene expression, which disrupts ribosome biogenesis, may be a trigger for p53 activation (264, 269, 270). Hematopoietic progenitor cells from a MYSM1-deficient human patient similarly had reduced protein synthesis rate and elevated p53 protein level (269). Together, these data demonstrate the crucial role of MYSM1 in maintaining hematopoiesis and suggest MYSM1 as a negative regulator of p53 stress response in hematopoietic cells (264-269). As the importance of MYSM1 in hematologic malignancies is currently unknown, the therapeutic potential of inhibiting MYSM1 for p53 activation in B-cell lymphoma is of great interest.

1.7 Ubiquitin-specific Protease 44 (USP44)

1.7.1 USP44 Protein Structure and Function

Ubiquitin-specific protease 44 (USP44) is a deubiquitinase from the USP family, consisting of a zinc-finger ubiquitin-binding (ZnF-UBP) domain at the N-terminus and a USP domain at the C-terminus (271). USP44 is capable of removing ubiquitin from histones H2A and H2B in the processes of DNA damage response and gene repression, respectively (178, 198, 200). In addition, USP44 deubiquitinates other protein substrates in the regulation of mitosis (272). "Both USP44 deficiency and overexpression predispose to errors in chromosome segregation, aneuploidy, and cancer (273, 274). *Usp44* knockout in mice results in increased susceptibility to lung adenomas (273), whereas in humans, *USP44* deletions or silencing are seen in lung and colorectal cancer (275, 276). In contrast, overexpression of *USP44* was observed in T-cell acute lymphoblastic leukemia (274), glioma (277), and gastric cancer (278)" (2).

1.7.2 USP44 in DNA Damage Response

As USP44-overexpression induced the deubiquitination of histone H2A, USP44 may prevent the formation of ubiquitin chains on H2A at the DNA double strand break (DSB) (178). Overexpression of USP44 showed normal accumulation of RNF8 but diffuse localization of RNF168 and RNF168 activity-dependent products (ubiquitin conjugates and RAP80) within the ionizing radiation-induced foci (178). USP44-overexpression also eliminated the formation of 53BP1 loci following irradiation, whereas knockdown of USP44 increased the levels of ubiquitin conjugates and 53BP1 at DSB regions (178). Interestingly, expression of a catalytically inactive version of USP44 showed the ability of USP44 to colocalize with γ-H2AX in a RNF8- and

RNF168-dependent manner (178). Altogether, these results suggested that USP44 associates with DSB and opposes the histone ubiquitination process that is recognized by RNF168, thereby preventing further ubiquitin-mediated recruitment of downstream factors to DNA damage sites (178). These dynamic processes of histone ubiquitination and deubiquitination control the extent of protein recruitment to the site of DNA damage (178).

1.7.3 USP44 in Gene Repression

USP44 also deubiquitinates H2B-K120ub in the regulation of embryonic stem cell differentiation (200). In the undifferentiated state, USP44 is needed to keep the H2B-K120ub levels low; in response to differentiation signals, USP44 is downregulated to enhance the levels of H2B-K120ub and contribute to the induction of RNF20-dependent gene transcription (200). USP44 has been shown to form complexes with other proteins (198). While interacting with subunits of the nuclear receptor co-repressor (N-CoR) complex, USP44 deubiquitinates H2B-K120ub at N-CoR target loci and participates in silencing gene expression (198). In addition, USP44 forms a complex with centrosome component centrin-2 (CETN2) and functions during mitosis, independently of the N-CoR complex (198).

1.7.4 USP44 in Cell Cycle and Mitosis

USP44 plays important functions during mitosis of the cell cycle, with studies demonstrating the significance of USP44 at two different time points (272, 273). One study showed USP44 participating in the spindle assembly checkpoint, in which the cell waits until all chromosomes are properly attached by spindles from bipolar ends before activating the anaphase-promoting complex (APC) (272). The APC is an E3 ubiquitin ligase that binds to cell division cycle 20 (Cdc20)

coactivator and, together as the APC^{Cdc20}, targets cell cycle proteins (e.g., securin and mitotic cyclins) for degradation, ultimately guiding sister chromatid separation during anaphase (272). When kinetochores of chromosomes are still unattached, mitotic arrest deficient 2 (Mad2) protein binds to Cdc20, inhibiting the function of APC^{Cdc20} (272). The expression of USP44 increases as mitotic cells arrest at the spindle checkpoint but decreases once cells complete and exit mitosis (272). USP44-deficient cells exhibited reduced Mad2-Cdc20 association and elevated APC^{Cdc20} activity, accompanied by accelerated anaphase onset and more frequent mitotic defects, such as the lack of a metaphase plate, the presence of unaligned and lagging chromosomes, and the absence of cytokinesis (272). These results suggested that USP44 stabilizes the interaction between Mad2 and APC^{Cdc20}, restraining early activation of APC^{Cdc20} to prevent premature separation of sister chromatids (272). As shown by using a catalytically inactive USP44, the catalytic activity is needed for USP44 to oppose APC function and maintain the spindle checkpoint function during mitosis, with potential deubiquitination targets including Cdc20 and/or other substrates of the APC (e.g., cyclin B and securin) (272).

Consistent with previous results, another study found that mouse embryonic fibroblasts (MEFs) derived from *Usp44*-knockout embryos exhibited accelerated mitosis from prophase to anaphase and increased chromosome missegregation errors (273). However, upon closer examination, mitotic defects in *Usp44*-knockout MEFs appeared early on, preceding the spindle checkpoint (273, 279). During prophase, before nuclear envelope breakdown occurred, centrosomes from *Usp44*-knockout MEFs often did not properly separate to direct, opposing ends of a cell, giving rise to merotelic attachment of kinetochore during prometaphase, in which spindle microtubules from both centrosomes erroneously attach to the same kinetochore of a chromosome (273, 279). During metaphase and anaphase, whereas normal chromosomes aligned at the midzone

and were pulled apart, with sister chromatids moving towards opposite poles, the chromosome with merotelically attached kinetochore would be left behind near the midzone, pulled in a tug-of-war fashion simultaneously by microtubules from both ends (279). As such, "lagging chromosomes" and abnormal chromosome numbers (aneuploidy) were more frequently observed in *Usp44*-knockout MEFs (273, 279). Further experiments showed that in this scenario, both the catalytic activity and the centrin-binding activity of USP44 were essential for the mitotic functions of USP44 (273). Nevertheless, the exact mechanism in which USP44 regulates chromosome segregation requires further investigation (279). As USP44 participates in gene transcription, DNA repair, cell cycle progression, as well as carcinogenesis, the role of USP44 in cellular development *in vivo* is of great interest.

1.8 Rationale and Objectives

Hematopoiesis is the process of generating mature blood cells from rare hematopoietic stem cells (HSCs) within the bone marrow through cell proliferation and differentiation (3). Following a series of developmental stages that require regulation of gene transcription, DNA repair, and cell cycle progression, B cells successfully complete V(D)J recombination and assemble surface B-cell receptors (4-6). Upon activation in peripheral lymphoid organs by T cells and antigen stimulation, B cells further proliferate and differentiate, emerging as important pathogen fighters of the adaptive immune response (3, 4). Dysregulations during the development of blood cells often associate with immunodeficiencies, bone marrow failure, and malignancies (4, 7, 8).

The process of hematopoiesis is tightly governed by transcription factors and chromatin-modifying enzymes (30). Despite its early discovery, histone (de)ubiquitination remains poorly understood (169, 170). The roles of histone deubiquitinases in mammalian physiology have only been recently unravelled (229, 230). Given the expression of three histone deubiquitinating enzymes (BAP1, MYSM1, and USP44) in blood cells and their functions in regulating gene transcription, DNA repair, and cell cycle progression, we hypothesized that they are essential in the hematopoietic and immune systems (280).

This thesis investigates the uncharacterized functions of:

(i) BAP1 in B-cell development,

(ii) MYSM1 in c-MYC-driven B-cell lymphoma, and

(iii) USP44 in HSC and B-cell functions.

CHAPTER 2: METHODOLOGY

The following methodology section contains paragraphs from the publications/ manuscripts:

- (Chapter 3.1) **YH Lin** et al., (2021) Regulation of B Lymphocyte Development by Histone H2A Deubiquitinase BAP1. *Frontiers in Immunology*. **12**:626418. (1)
- (Chapter 3.2) **YH Lin** et al., Loss of MYSM1 inhibits the oncogenic activity of cMYC in B cell lymphoma. (Manuscript accepted in the *Journal of Cellular and Molecular Medicine*)
- (Chapter 3.3) **YH Lin** et al., (2019) USP44 is dispensable for normal hematopoietic stem cell function, lymphocyte development, and B-cell-mediated immune response in a mouse model. *Experimental Hematology*. **72**, 1-8. (2)

2.1 Mouse Strains

(Chapter 3.1) "*Bap1*$^{tm1c(EUCOMM)Hmgu}$ mouse strain carries a floxed (conditional) allele of *Bap1*-gene and will be referred to here as *Bap1*^{fl}. The strain is derived from ES-cell clone HEPD0526_2_G01; allele structure is provided at *https://www.mousephenotype.org/data/alleles/MGI:1206586/tm1c(EUCOMM)Hmgu*, and the mice are available from EMMA at: *https://www.infrafrontier.eu/search?keyword=Bap1*. Specifically, *loxP* sites flank exons 6-12 (ENSMUSE00000121807- ENSMUSE00000121801) of the *Bap1* transcript ENSMUST00000022458.10. We thank the Wellcome Trust Sanger Institute Mouse Genetics Project, its funders, and INFRAFRONTIER/EMMA (*www.infrafrontier.eu*) for providing this strain. Funding information is found at *www.sanger.ac.uk/mouseportal* and associated primary phenotypic information at *www.mousephenotype.org* (281-284). The strain was bred to a transgenic line expressing Cre recombinase under the control of a B cell lineage specific promoter mb1-*Cre* (from Prof. Michael Reth, MPI für Immunbiologie und Epigenetik, Germany)

(285). The *Bap1*$^{\Delta}$ allele is predicted to disrupt *Bap1* protein coding sequence from amino acid 126 onward, precluding the expression of full N-terminal UCH catalytic domain and all downstream domains of BAP1 protein. All lines were on the C57BL/6 genetic background. The mice were maintained under specific pathogen-free conditions. Test and control groups were bred as littermates and co-housed together. Experiments were in accordance with the guidelines of the Canadian Council on Animal Care and protocol AUP-2011-6029 approved by the McGill Animal Care Committee. Mice used in experiments were both male and female, 8-16 weeks of age, and always age-matched and sex-matched between the experimental groups. Genotyping was performed with DreamTaq DNA Polymerase (Thermo Fisher Scientific) and primers from IDT Technologies" (1).

(Chapter 3.2) Mouse line B6.Cg-Tg(IghMyc)22Bri/J, also known as *Eμ-Myc*, is a widely studied model of B cell lymphoma and overexpresses cMYC under the control of the immunoglobulin heavy chain locus enhancer (286). Mouse lines *Mysm1*$^{-/-}$ and *Mysm1*$^{fl/fl}$ carry the loss-of-function and the conditional alleles of *Mysm1* gene, respectively, and were previously described (230, 267, 282). *Mysm1*$^{fl/fl}$*Cre*^{ERT2} mice were derived for tamoxifen-induced *Mysm1*-gene deletion by crossing the *Mysm1*$^{fl/fl}$ and Gt(ROSA)26Sor$^{tm1(cre/ERT2)}$ strains, as previously described (267). All lines were on the C57BL/6 genetic background. The mice were maintained under specific pathogen-free conditions. All experiments were in accordance with the guidelines of the Canadian Council on Animal Care, and protocol AUP-7643 approved by the McGill University Animal Care Committee.

(Chapter 3.3) "The *Usp44*$^{tm1b(EUCOMM)Hmgu}$ mouse line, also known as *Usp44*$^{-/-}$, was generated by the Mouse Genetics Program of the Wellcome Trust Sanger Institute for the International Mouse Phenotyping Consortium (IMPC) (281, 282) and carries deletion of *Usp44*

exon *ENSMUSE00001396196*. The mouse line is therefore derived independently from the previously described *Usp44*$^{tm1.2Pjgl}$ strain (273), but it carries deletion of the same exon and therefore is expected to be functionally equivalent. The deleted exon encodes the first 475 out of 711 amino acids of USP44 protein, including the catalytic site. Information on the *Usp44*$^{-/-}$ allele structure and its validation conducted by the IMPC mouse production pipeline is available online at: www.infrafrontier.eu/search?keyword=Usp44; www.mousephenotype.org/about-ikmc/targeting-strategies; and https://mpi2.github.io/IKMC-knowledgebase/ikmc/screens_and_quality_control/wtsi_mouse_clinic_quality_control_tests__mice. Allele structure was additionally validated in this study through extensive *Usp44* locus genotyping, sequencing, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses (Figure 3.3.1 and Tables 3.3.2-3.3.3). *Usp44*$^{-/-}$ mice were crossed with the B6.Cg-Tg(IghMyc)22Bri/J mouse model of B-cell lymphoma (Eμ-Myc, JAX: 002728) (286). All lines were maintained on the C57BL/6 background under specific pathogen-free conditions. All procedures were in accordance with the guidelines of the Canadian Council on Animal Care and the animal use protocol approved by the McGill University Animal Care Committee" (2). "Mouse samples were genotyped with PCR using DreamTaq DNA Polymerase (Fisher Scientific) and primers purchased from Integrated DNA Technologies, with primer sequences provided in Table 3.3.2. Sanger sequencing of PCR products, where applicable, was performed at the core facilities of McGill University and Genome Quebec Innovation Centre" (2).

2.2 Bone Marrow Transplantation

(Chapter 3.1) "For competitive bone marrow transplantations, recipient B6.SJL-PtprcaPepcb/Boy mice (JAX002014, congenic for CD45.1) were irradiated with 2 doses of 4.5Gy, delivered 3 hours

apart, in a RS2000 irradiator (Rad Source). Wild-type CD45.1-marked bone marrow cells were mixed in a 1:1 ratio with bone marrow cells from mice of *Bap1*$^{fl/+}$, *Bap1*$^{fl/+}$*Cre*, or *Bap1*$^{fl/fl}$*Cre* genotypes, and the mixes transplanted into three independent cohorts of recipient mice. The mice were kept on neomycin in drinking water (2g/l, BioShop) for 3 week, and analyzed at 10 and 17 weeks post-reconstitution" (1).

(Chapter 3.3) "Recipient mice were lethally irradiated with two doses of 4.5 Gy in an RS2000 irradiator (Rad Source) and injected intravenously with 2 million donor bone marrow cells. For the primary transplantation, the donor cells consisted of a 1:1 mixture of CD45.1$^+$ and CD45.2$^+$ bone marrow cells, with the CD45.2$^+$ cells derived either from the control *Usp44*$^{+/+}$ or the knockout *Usp44*$^{-/-}$ mice. The primary recipients were analyzed at >20 weeks after reconstitution, and the experiment was repeated twice with at least four recipient mice per group per experiment. For the secondary transplantation, 2 million donor bone marrow cells harvested from the primary recipients were transplanted into new groups of lethally irradiated recipient mice and these secondary recipients were analyzed after further >20 weeks" (2).

2.3 Flow Cytometry

(Chapter 3.1) "Cell suspensions of mouse tissues were prepared in RPMI-1640 (Wisent) with 2% (v/v) FCS, 100μg/ml streptomycin and 100U/ml penicillin (Wisent). The cells were stained for surface-markers in PBS with 2% FCS for 20 minutes on ice, with the following antibodies: APC-conjugated anti-CD43 (S7, BD Biosciences) or anti-IgD (11-26c.2a, BioLegend); APC-eFluor 780-conjugated anti-CD45R/B220 (RA3-6B2, eBioscience) or anti-CD45.1 (A20, eBioscience); Brilliant Violet 650-conjugated anti-CD45R/B220 (RA3-6B2, BioLegend); BUV395-conjugated anti-CD43 (S7, BD Biosciences); eFluor 450-conjugated anti-CD24 (M1/69, eBioscience) or anti-

CD45R/B220 (RA3-6B2, eBioscience); FITC-conjugated anti-CD23 (B3B4, eBioscience), anti-CD127/IL7Rα (A7R34, eBioscience), or anti-CD249/BP-1 (6C3, eBioscience); Pacific Blue-conjugated anti-IgD (11-26c.2a, BioLegend); PE-conjugated anti-IgM (II/41, eBioscience); PE-Cy7-conjugated anti-CD19 (6D5, BioLegend), anti-CD21/CD35 (eBio8D9, eBioscience) or anti-CD45.2 (104, BioLegend); PerCP-Cy5.5-conjugated anti-CD19 (1D3, Tonbo Biosciences) or anti-CD45R/B220 (RA3-6B2, BioLegend)" (1).

(Chapter 3.1) "Intracellular staining was performed as previously described (264, 268). The cells were fixed in 2% paraformaldehyde (PFA) in PBS with 2% FCS at 37℃ for 10 minutes, and permeabilized in 90% methanol for 30 minutes on ice. The cells were then stained with Alexa Fluor 647-conjugated anti- p53 (1C12, Cell Signaling Technology) and Alexa Fluor 488-conjugated anti-H2A.X Phospho (Ser139) (2F3, BioLegend) antibodies, or FITC-conjugated anti-Ki-67 (B56, BD Biosciences) and Alexa Fluor 647-conjugated Histone H3 Phospho (Ser10) (11D8, BioLegend) antibodies, or the appropriate isotype controls. Viability Dye eFluor® 506 (eBioscience) was used to discriminate dead cells. Annexin V PeCy7 (eBioscience) was used for detection of apoptotic cells. Compensation was performed with BD™ CompBeads (BD Biosciences). The data were acquired on FACS Canto II or BD Fortessa and analyzed with FACS Diva (BD Biosciences) or FlowJo (Tree Star) software" (1).

(Chapter 3.2) Cell suspensions of mouse spleen and lymph nodes were prepared in 45% DMEM (Life Technologies), 45% IMEM (Life Technologies), 10% FCS (Wisent), 100μg/ml streptomycin and 100U/ml penicillin (Wisent), and $5x10^{-5}$ M β-mercaptoethanol (Sigma-Aldrich). The cells were stained for surface-markers in PBS with 2% FCS for 20 minutes on ice with eFluor450-conjugated anti-CD45R/B220 (RA3–6B2, BioLegend). Fixable Viability Dye

eFluor506 (eBioscience) was used to discriminate dead cells, and compensation done with BD™ CompBeads (BD Biosciences).

(Chapter 3.2) Intracellular straining for flow cytometry was performed as previously described (264, 268). The cells were fixed in 2% paraformaldehyde (PFA) in PBS with 2% FCS at 37℃ for 10 minutes, and permeabilized in 90% methanol for 30 minutes on ice. The cells were stained with intracellular antibodies: Alexa Fluor 488 anti-p53 (clone 1C12, Cell Signaling), or unconjugated anti-cMYC (clone D84C12, Cell Signaling) or anti-EEF1G (EPR7200, Abcam) with Alexa Fluor 488 anti-rabbit IgG highly cross-adsorbed secondary antibody (Life Technologies), or appropriate isotype controls. All data were acquired on FACS Canto II flow cytometer (BD Biosciences) and analyzed with FACS Diva (BD Biosciences) or FlowJo (Tree Star) software.

(Chapter 3.3) "Flow cytometry was performed as previously described (266, 287). Cell suspensions of mouse tissues were prepared in RPMI-1640 (Wisent) with 2% (v/v) Fetal Calf Serum (FCS), 100μg/ml streptomycin and 100U/ml penicillin (Wisent), and subjected to red blood cell lysis in Ammonium-Chloride-Potassium (ACK) buffer. Cells were stained for surface-markers in PBS with 2% FCS and 0.2% (w/v) sodium azide (VWR, Amresco) for 20 minutes on ice" (2).

(Chapter 3.3) "The following fluorophore-conjugated antibodies were used in different combinations: FITC antibodies against CD23 (B3B4, eBioscience), CD34 (RAM34, eBioscience), CD48 (HM48-1, eBioscience), and NK1.1 (PK136, BioLegend); PE antibodies against CD25 (PC61.5, eBioscience), CD135/FLT3 (A2F10, BioLegend), CD150 (mShad150, eBioscience), and IgM (II/41, eBioscience); PerCP/Cy5.5 antibodies against CD4 (RM4-5, BioLegend), CD8a (53-6.7, BioLegend), CD11b (M1/70, BioLegend), CD19 (1D3, Tonbo Biosciences), CD45R/B220 (RA3- 6B2, BioLegend), and GR1 (GR1, BioLegend); APC antibodies against CD8a (53-6.7, BioLegend), CD44 (IM7, BioLegend), IgD (11-26c.2a, BioLegend), and Sca-1 (E13-161.7,

BioLegend); Pacific Blue antibodies against CD11b (M1/70, BioLegend) and CD117/cKit (2B8, BioLegend); eFluor450 antibodies against CD8 (53-6.7, eBioscience) and CD45/B220 (RA3-6B2, eBioscience); PE-Cy7 antibodies against CD4 (RM4-5, BioLegend), CD21/CD35 (eBio8D9, eBioscience), CD45.2 (104, BioLegend), and CD117/cKit (2B8, BD Biosciences); APC-Cy7 antibodies against: CD45 (30-F11, BioLegend), CD45.1 (A20, BioLegend), and MHCII (M5/114.15.2 BioLegend)" (2).

(Chapter 3.3) "Specifically, for the analysis of *in vitro* cell activation, cells were stained with anti-mouse B220- PerCP-Cy5.5 (RA3-6B2, BioLegend), CD54-FITC (YN1/1.7.4), CD80-PE (16-10A1), CD86- APC (GL1, all from eBioscience), and MHCII-APC-Cy7 (M5/114.15.2, BioLegend). For analysis of immunoglobulin class switching, the cells were stained with goat anti-mouse IgG-PE (Southern Biotech), followed by B220-PerCP-Cy5.5 (RA3-6B2, BioLegend) and IgD (11-26c.2a, BioLegend). For analysis of cell proliferation, cells were pre-loaded with CellTrace CFSE dye (Life Technologies, Fisher Scientific), according to the manufacturer's recommendations" (2).

(Chapter 3.3) "Fixable Viability Dye eFluor506 (eBioscience) was used to discriminate dead cells. Compensation was performed with BD CompBeads (BD Biosciences). The data were acquired on FACS Canto II and analyzed with FACS Diva (BD Biosciences) or Flowjo (Flowjo LLC) software" (2).

2.4 B Cell Isolation and Cell Sorting

(Chapter 3.1) "Total B cells were isolated from mouse spleen via magnetic enrichment using EasySep Mouse CD19 Positive Selection Kit II (Stem Cell Technologies), according to the manufacturer's protocols. B cell subsets were sorted from mouse bone marrow, as previously

described (264). Bone marrow was flushed in PBS supplemented with 0.1% BSA and 2mM EDTA, filtered through 40μm cell-strainers, and subjected to red blood cell lysis in ACK buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA). The samples were stained with: biotin-conjugated antibodies against lineage markers [anti-CD3ε (145-2C11, BioLegend), anti-CD11b (M1/70, BioLegend), anti-CD11c (N418, BioLegend), anti-Ly-6G/Ly-6C (Gr-1) (RB6-8C5, BioLegend), anti-NK-1.1 (PK136, BioLegend), and anti-TER-119 (TER-119, BioLegend)], followed by APC-eFluor 780-conjugated Streptavidin (eBioscience), Alexa Fluor 488-conjugated anti-IgD (11-26c.2a, BioLegend), APC-conjugated anti-CD43 (S7, BD Biosciences), PE-conjugated anti-IgM (II/41, eBioscience), PE-Cy7-conjugated anti-CD19 (6D5, BioLegend), and PerCP-Cy5.5-conjugated anti-CD45R/B220 (RA3-6B2, BioLegend) antibodies. DAPI was added immediately before sorting for dead cell exclusion. Cell sorting was performed on FACSAria and analyzed with FACS Diva software (BD Biosciences)" (1).

(Chapter 3.2) Cell sorting was performed on FACSAria II (BD Biosciences), with cells pre-stained with PE anti-IgM (II/41, eBioscience), PerCP-Cy5.5 anti-CD45R/B220 (RA3–6B2, BioLegend), and DAPI to discriminate dead cells.

(Chapter 3.3) "B cells were isolated from mouse bone marrow or spleen using magnetic enrichment with the EasySep Mouse CD19 Positive Selection Kit II (Stem Cell Technologies)" (2).

2.5 Pre-B Colony Forming Unit (CFU) Assays

(Chapter 3.1) "Pre-B Colony Forming Unit (CFU) assays with primary mouse bone marrow were performed according to manufacturer's protocols using MethoCult M3630 media (StemCell Technologies), with $1x10^5$ mouse bone marrow cells plated per dish" (1).

2.6 Western Blotting

(Chapter 3.1) "Western blotting was performed as previously described (264). Magnetically isolated murine B cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Thermo Scientific). Protein concentration was measured using the BCA assay (Thermo Scientific). Protein lysate samples were boiled in Laemmli buffer and 1.25% β-mercaptoethanol (Sigma-Aldrich) before loading onto gels, alongside Precision Plus Protein Kaleidoscope standards (Bio-Rad). Upon gel-to-membrane transfer, nitrocellulose membranes (GE Healthcare) were blocked with 5% milk in TBS-T and probed with rabbit monoclonal primary antibodies against BAP1 (D7W7O, Cell Signaling Technology) and β-Actin (D6A8, Cell Signaling Technology) at 4°C overnight, followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit polyclonal IgG secondary antibody (Abcam) at room temperature for 1 hour, with TBS-T washes after each antibody-incubation. Protein bands were detected using Western Lightning Plus-ECL (PerkinElmer) and HyBlot CL autoradiography films (Harvard Apparatus Canada), according to manufacturer's protocol" (1).

2.7 RNA Isolation and qRT-PCR

(Chapter 3.1) "RNA isolation from magnetically isolated B cells was carried out with the EZ-10 DNAaway RNA mini-prep kit (BioBasic) according to the manufacturer's protocol. The RNA was quantified with Nanodrop spectrophotometry (ThermoFisher Scientific), and reverse transcribed with the M-MLV reverse-transcription kit (Life Technologies, ThermoFisher Scientific). All qPCRs were performed on a StepOnePlus instrument with Power SYBR Mastermix (Applied Biosystems, Life Technologies). The primer sequences for validating *Bap1*-depletion in murine B cells were: *Bap1* -Fw GGA TTG AAA GTC TAC CCA ATT GAT, *Bap1* -Rv CGA GCT TTA

TCT GTC CAC TCC T, *Actb* -Fw CTA AGG CCA ACC GTG AAA AG, *Actb* -Rv ACC AGA GGC ATA CAG GGA CA; all primers were purchased from IDT Technologies" (1).

(Chapter 3.2) RNA isolation from cell lines was carried out using the MagMAX total RNA kit (Ambion, Life Technologies) according to the manufacturer's protocol. RNA quality was assessed on Bioanalyzer RNA Pico chips (Agilent), and cDNA was prepared using the qScript XLT cDNA Supermix (Quanta Biosciences) with 2-5ng RNA input per reaction. qPCRs were performed on a StepOnePlus instrument with Power SYBR Mastermix (Applied Biosystems, Life Technologies). The primers were purchased from IDT Technologies, with sequences below:

Table 2.7.1 for Chapter 3.2: RT-qPCR Primers Sequences.

Target Gene	Forward Sequence	Reverse Sequence
Rps3	ctgaaggcagcgtagagctt	tccaaggagtttgtagcgtaga
Rps10	gtgagcgacctgcaagattc	cagcctcagctttcttgtca
Rps24	gcagtgagcggtcctcttt	ggtccggatggttactgtgt
Rpl7	ccttgattgctcggtctctt	agcctgtttatctggtcttcc
Rpl9	catccaggagaatggctcttt	cagttcccttctcagacacatag
Rpl11	aaatgagaagattgctgttcactg	caactcatactcccgcacct
Rpl13	gaaacaagtccacggagtca	ttgctcggatgccaaaga
Eef1g	tcacgagaggagaaacagaaac	cagggaccagccatctttatc
Hprt	caggccagactttgttggat	ttgcgctcatcttaggcttt
Mysm1	gggattccgacctaacttgtc	tggaaaggaacagattttctatttg

(Chapter 3.3) "RNA was isolated with MagMAX total RNA kit (Ambion, Life Technologies), and reverse transcribed with M-MLV reverse-transcription kit (Invitrogen, Life Technologies). qRT-PCRs were performed on a StepOnePlus instrument with Power SYBR Mastermix (Applied Biosystems, Life Technologies). The primers were purchased from IDT Technologies, and the sequences are provided in Table 3.3.3" (2).

2.8 RNA Sequencing (RNA-Seq)

(Chapter 3.1) "The protocols were as previously described (264). Briefly, RNA was isolated using the MagMAX total RNA kit (Ambion, Life Technologies), and quality assessed using Bioanalyzer RNA Pico chips (Agilent). rRNA depletion and library preparation were performed using the SMARTer Stranded RNA-Seq kit (Takara Clontech). The libraries were sequenced on an Illumina HiSeq 4000 sequencer in paired-end 50bp configuration. The high quality of sequence reads was confirmed using the FastQC tool (Babraham Bioinformatics), and low-quality bases were trimmed from read extremities using Trimmomatic v.0.33 (288). Reads were then mapped to the mouse UCSC mm9 reference assembly using TopHat v2.0.9 in conjunction with Bowtie 1.0.0 algorithm (289-291). Gene expression was quantified by counting the number of uniquely mapped reads with featureCounts using default parameters (292). We retained genes that had an expression level of minimum 5 counts per million reads (CPM) in at least 3 of the samples, and performed quantile normalization with the preprocessCore package to remove batch effects (293). TMM normalization and differential gene expression analyses were conducted using the edgeR Bioconductor package (294). Dimension reduction analysis was performed using the Partial Least Square regression method (295). Pairwise comparisons were performed between samples across different mouse genotypes. Genes with changes in expression $\geq |1.5|$ folds and Benjamini-Hochberg adjusted p values ≤ 0.01 across the three genotypes in either pre-B or immature B cells were considered significant. For data visualization in Integrative Genomics Viewer (IGV) (296), replicates with the same genotype were combined, and bigwig files were generated using a succession of genomeCoverageBed and wigToBigWig tools and scaled per 10 million exon-mapped reads. Gene ontology (GO) and disease ontology enrichment analyses on differentially expressed gene clusters were performed with DAVID Bioinformatics Resources 6.8 (297), and

Gene Set Enrichment Analysis (GSEA) was performed in command-line using MSigDB v6.0 with default configuration and permutation within gene sets (298)" (1).

2.9 Tamoxifen Mouse Treatment

(Chapter 3.2) For tamoxifen-induced *Mysm1*-gene deletion, mice of *Mysm1*$^{fl/fl}$*Cre*^{ERT2} and control genotypes were injected intraperitoneally with tamoxifen (Sigma-Aldrich, T5648) in sterilized corn oil at 0.15 mg/gram per injection, with 8 doses administered in total over 16 days. Successful deletion of *Mysm1* exon 3 was validated by PCR analyses of the genomic DNA from hematopoietic and lymphoid organs of the mice, and the loss of *Mysm1* transcript was further confirmed by qRT-PCR with Lin$^-$cKit$^+$Sca1$^+$ hematopoietic stem and progenitor cells isolated from the mouse bone marrow, as described previously (264, 267). Control animals of the same genotypes injected with vehicle corn oil (Sigma-Aldrich) were also included in the analyses.

2.10 Adoptive Transfer of B Cell Lymphoma Cells

(Chapter 3.2) Tumour cells harvested from mice of *Eμ-Myc Mysm1*$^{fl/fl}$*Cre*^{ERT2} and control *Eμ-Myc Mysm1*$^{fl/fl}$ genotypes were subjected to red blood cell lysis in ACK buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA) and cryopreserved, then subsequently transferred into recipient wild type C57BL/6 mice subjected to 3.5 Gy of irradiation, at 10^6 cells per recipient via an intravenous injection. The recipients were administered with tamoxifen or vehicle corn oil, as described above (264, 267), and mouse health and survival monitored over subsequent 100 days.

2.11 Culture of Primary Cells

(Chapter 3.2) Tumour cells harvested from spleen and lymph nodes of Eμ-Myc mice of different *Mysm1* genotypes were cultured on a monolayer of irradiated *Ink4a*$^{-/-}$ mouse embryonic fibroblasts (MEFs) in media containing 45% DMEM (Life Technologies), 45% IMEM (Life Technologies), 10% FCS (Wisent), 100μg/mL streptomycin and 100U/mL penicillin (Wisent), and $5x10^{-5}$ M β-mercaptoethanol (Sigma-Aldrich).

2.12 Protein Synthesis Rate Measurements

(Chapter 3.2) Analysis of protein synthesis rates was performed using the O-propargyl-puromycin (OPP) incorporation method. Briefly, cells were cultured in the presence of 20 μM OPP for 30 minutes, stained with Fixable Viability Dye eFluor506 (eBioscience), fixed in 2% paraformaldehyde (PFA) in PBS with 2% FCS at 37℃ for 10 minutes, and permeabilized in 90% methanol for 30 minutes on ice. The cells were then washed with PBS, and staining for OPP incorporation using the Click-iT™ Plus OPP Alexa Fluor 488 Protein Synthesis Assay Kit (Life Technologies, Thermo Fisher Scientific) according to the manufacturer's protocols. Samples were analyzed by flow cytometry on FACS Canto II with FACS Diva software (BD Biosciences).

2.13 Whole-body Irradiation

(Chapter 3.3) “Mice were irradiated with a single dose of 6 or 7 Gy in an RS2000 irradiator (Rad Source) and their survival was monitored” (2).

2.14 Mouse Immunization

(Chapter 3.3) "Mouse immunizations, followed by ELISA measurements of antigen-specific serum antibody titers, were performed as described by Förster et al. (266). Mice were immunized subcutaneously in both flanks with immunogen emulsion containing: 100μL of PBS, 100μL of Complete Freund's Adjuvant (CFA, Fisher Scientific) and 15μg of R-Phycoerythrin (ProZyme), vortexed for 45 minutes prior to injection. Serum was collected from the mice at day 14 after immunization to measure antigen specific antibody titres" (2).

2.15 ELISA

(Chapter 3.3) "To measure the antigen-specific antibody titres in mouse serum, plates were coated with R-Phycoerythrin (ProZyme, 10µg/mL), and following incubation with serial dilutions of the serum samples, developed with biotin goat anti-mouse IgM, IgG1, IgG2c and IgG3 antibodies (Southern Biotech), followed by Streptavidin-Peroxidase (R&D Systems) and SuperAquaBlue Substrate (eBioscience, Fisher Scientific). Plates were imaged with an EnSpire 2300 Multilabel Reader (Perkin Elmer)" (2).

2.16 *In vitro* B-cell Stimulation Assays

(Chapter 3.3) "The assays were performed as described by Förster et al. (266). B cells were isolated from mouse spleen using magnetic enrichment, with either CD45R (B220) MicroBeads (Miltenyi Biotec) or EasySep Mouse CD19 Positive Selection Kit II (Stem Cell Technologies), following the manufacturers' protocols. B cells were plated at $1x10^6$ cells/mL in RPMI-1640 with 10% (v/v) fetal calf serum (FCS), 2mM L-glutamine, 1mM sodium pyruvate, 100 µg/ml streptomycin, 100U/ml penicillin (all from Wisent), and $5x10^{-5}$ M β-mercaptoethanol (Sigma-Aldrich). The cells

were stimulated with LPS (Millipore 1 μg/mL), or anti-CD40 (eBioscience, 2 μg/mL) + IL4 (Peprotech, 5 ng/mL). For analysis of cell proliferation, the cells were pre-loaded with CellTrace CFSE dye (Life Technologies, Fisher Scientific), according to the manufacturer's recommendations" (2).

2.17 Statistical Analyses

(Chapter 3.1) "Statistical analyses used Prism 7.01 (GraphPad Inc.), with Student's *t*-test for two datasets and ANOVA with Sidak's or Tukey's post hoc tests for multiple comparisons" (1).

(Chapter 3.2) Statistical comparisons were performed with Prism 7.01 (GraphPad), using Student's *t*-test for two groups, ANOVA with Bonferroni post-hoc test for multiple comparisons, and Kaplan–Meier regression analysis for survival data.

(Chapter 3.3) Statistical analyses were performed as written for Chapter 3.2.

CHAPTER 3: RESULTS

3.1 ROLE OF BAP1 IN B LYMPHOCYTE DEVELOPMENT

3.1.1 Depletion of B cells in the *Bap1*$^{fl/fl}$ *Cre* mouse model

"To study the role of BAP1 in the B cell lineage, a B cell specific conditional knockout mouse model was generated by crossing the *Bap1*-floxed mouse line *Bap1*$^{fl/fl}$ to the mb1-*Cre* line expressing *Cre*-recombinase under the control of the B cell lineage specific *Cd79a* gene promoter, from the pre-pro-B cell stage in development (285). As expected, *Bap1*$^{fl/fl}$ *Cre* offspring were born in normal Mendelian ratios, showed no gross morphological abnormalities, and bred normally. Loss of BAP1 transcript and protein expression was validated in B cells isolated from the spleen of *Bap1*$^{fl/fl}$ *Cre* and control mice, using qRT-PCR and Western blotting, respectively (Figure 3.1.1A-B)" (1).

"To assess the effects of BAP1-loss on the B cell lineage, spleens and mesenteric lymph nodes of *Bap1*$^{fl/fl}$ *Cre*, *Bap1*$^{fl/+}$ *Cre*, and control *Bap1*$^{fl/+}$ mice were analyzed by flow cytometry, gating on CD19^{+} B cells. A significant reduction in the frequency of B cells was observed in the *Bap1*$^{fl/fl}$ *Cre* relative to control mice in both tissues (Figure 3.1.1C-E). The absolute numbers of B cells were also significantly reduced in the spleen of *Bap1*$^{fl/fl}$ *Cre* mice and showed a trend toward reduction in the lymph nodes (Figure 3.1.1C-E). We further confirmed the depletion of CD19^{+}B220^{hi} B2 lymphocytes in the spleen of *Bap1*$^{fl/fl}$ *Cre* mice, while CD19^{+}B220^{lo} cells were strongly expanded (Figure 3.1.2A-C)" (1).

Figure 3.1.1 Loss of BAP1 and depletion of B cells in the *Bap1*$^{fl/fl}$ *Cre* mouse model.

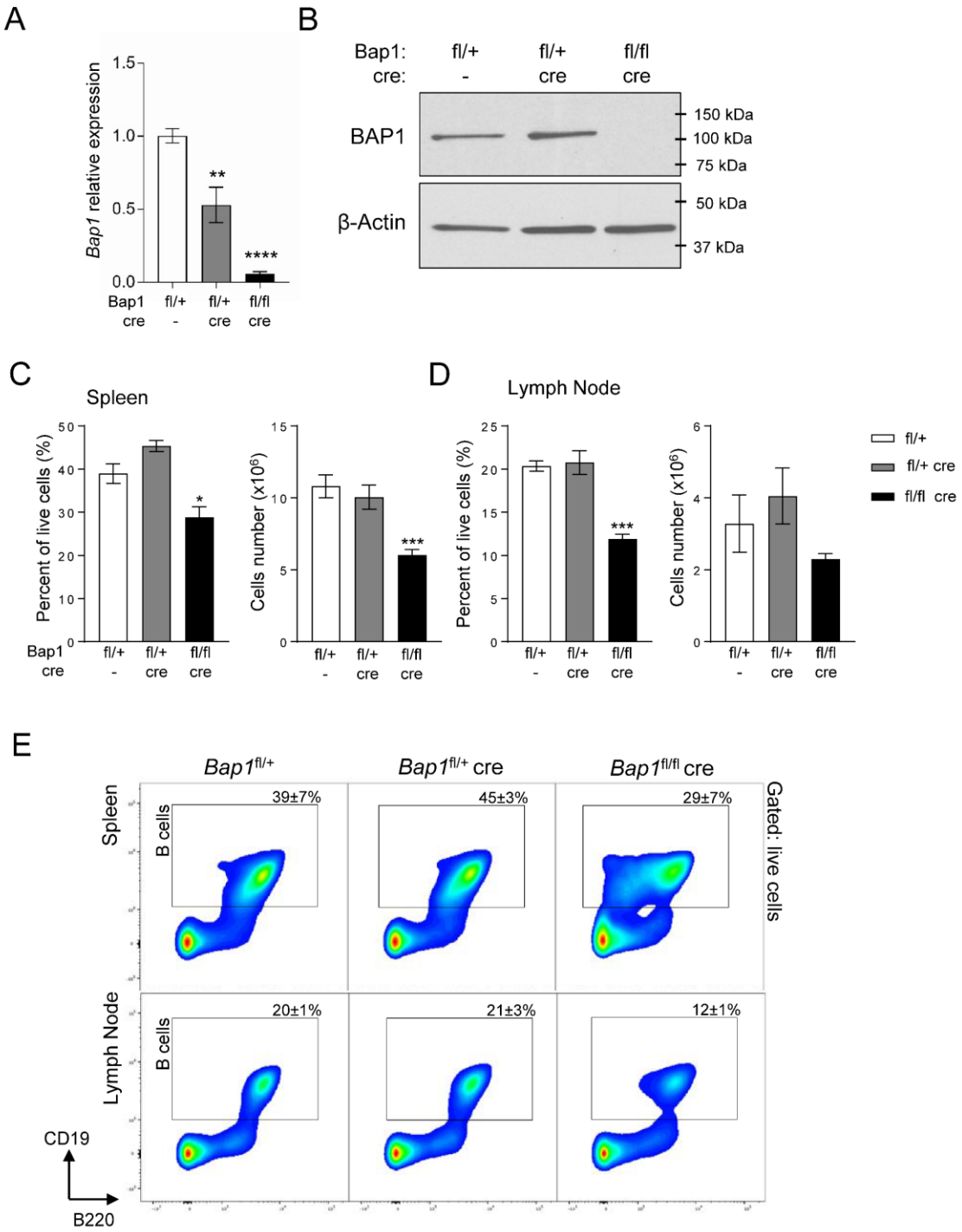
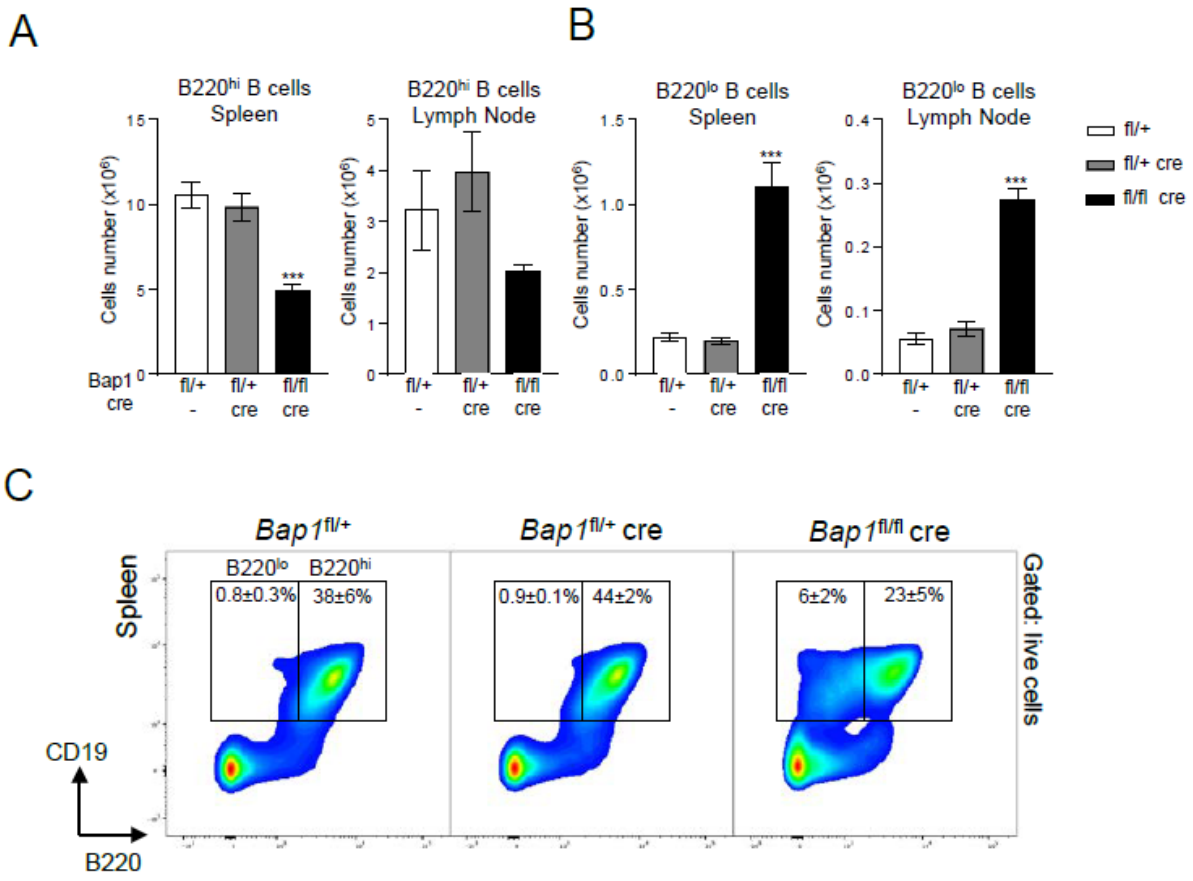


Figure 3.1.1 Loss of BAP1 and depletion of B cells in the *Bap1*$^{fl/fl}$ *Cre* mouse model.

"**(A)** Reduced *Bap1* transcript levels in B cells magnetically isolated from the total splenocytes of *Bap1*$^{fl/fl}$ *Cre*, relative to control mice, analyzed by RT-qPCR. Transcript levels are normalized to β-actin, and expressed relative to the transcript levels in control B cells. Data is from 4 mice per group; bars represent mean±SEM; statistical analysis by ANOVA, ** $p<0.01$, **** $p<0.0001$. **(B)** Loss of BAP1 protein in B cells magnetically isolated from the total splenocytes of *Bap1*$^{fl/fl}$ *Cre* relative to control mice, analyzed by Western blotting, using β-actin as the loading control; data from one mouse per genotype. **(C-D)** Depletion of B cells in the spleen (C) and mesenteric lymph nodes (D) of *Bap1*$^{fl/fl}$ *Cre* relative to control mice, expressed as the percentage of $CD19^+$ cells within the total live cells from the tissue and as the absolute cell number. Data presented is from two experiments with 4 mice analyzed per group per experiment. Bars represent mean±SEM; statistical analysis by ANOVA, NS non-significant, *$p<0.05$, *** $p<0.001$. **(E)** Representative flow cytometry density plots of the spleen (top) and mesenteric lymph nodes (bottom) stained for B cell lineage markers B220 and CD19, and gated on live cells. $CD19^+$ B cell gate is shown and the percentage of cells within the gate for each mouse genotype is presented as mean ± S.D." (1).

Figure 3.1.2 Analysis of B2 and B1 cell populations in *Bap1*$^{fl/fl}$ *Cre* mice.



"**(A-B)** Absolute numbers of CD19^{+}B220^{hi} and CD19^{+}B220^{lo} B lymphocytes in the spleen and mesenteric lymph nodes of *Bap1*$^{fl/fl}$ *Cre* and control mice. **(C)** Representative flow cytometry plots of the spleen of *Bap1*$^{fl/fl}$ *Cre* and control mice, gated on live cells, and showing CD19^{+}B220^{hi} and CD19^{+}B220^{lo} B lymphocyte gates. Percentages of cells within each gate are presented as mean ± S.D. Data is from 3-5 mice per genotype per experiment, with 2 experiments performed. Percentages of cells within each gate are presented as mean ± S.D. Bars represent mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, comparisons by *t*-test for two groups and by ANOVA for multiple comparisons" (1).

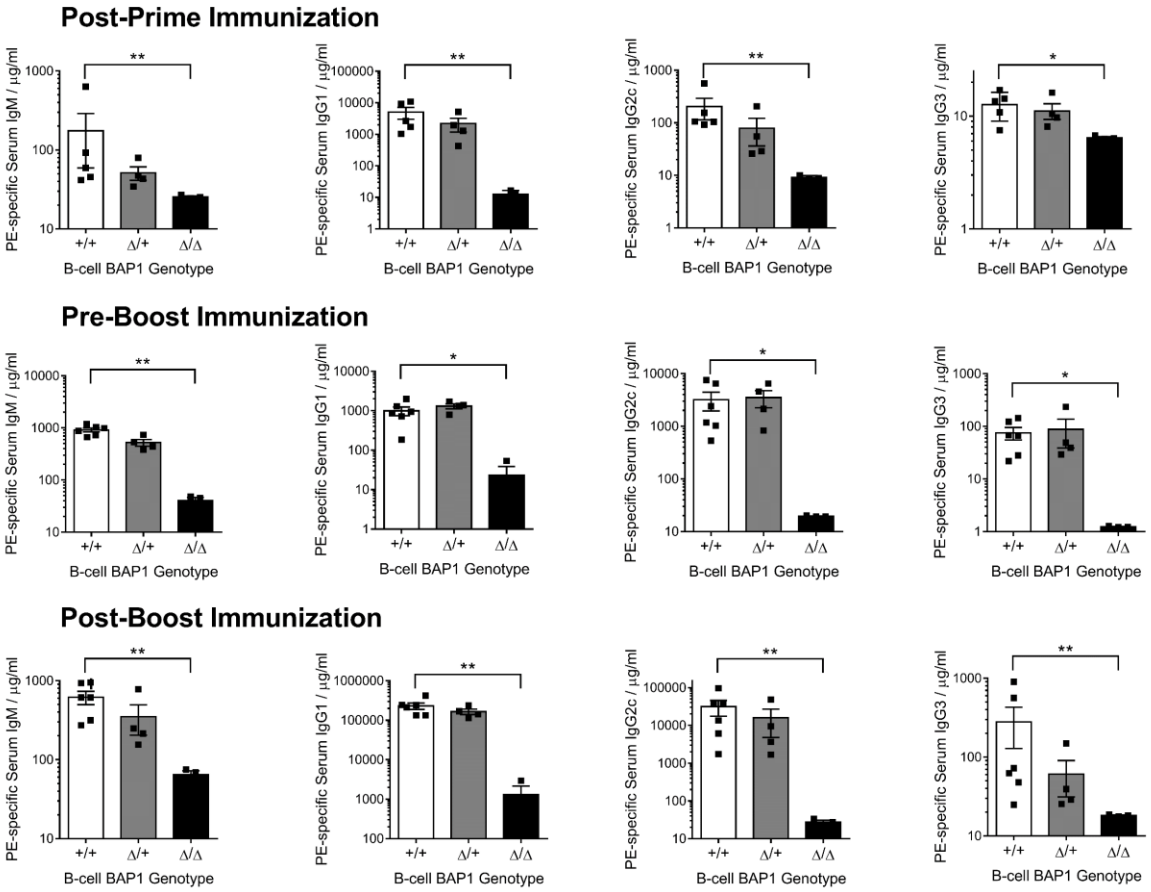
3.1.2 Reduction of antigen-specific antibody titers in the *Bap1*$^{fl/fl}$ *Cre* mouse model

To examine the effects of BAP1-loss on antigen-specific antibody response, *Bap1*$^{fl/+}$, *Bap1*$^{fl/+}$ *Cre*, and *Bap1*$^{fl/fl}$ *Cre* mice were immunized with a fluorescent R-phycoerythrin (R-PE) antigen dissolved in Complete Freund's Adjuvant (CFA) and PBS. Serum was collected 14 days after the prime immunization ("post-prime"), before the boost immunization ("pre-boost"), and 7 days after the mice were boosted with another dose of R-PE antigen in CFA/ PBS ("post-boost"). Antigen-specific antibody titers in the serum of *Bap1*$^{fl/fl}$ *Cre* mice after prime and boost immunizations were strongly reduced compared to those of control mice, as measured by sandwich ELISA, demonstrating that *Bap1*$^{fl/fl}$ *Cre* mice were impaired in the production of antigen-specific antibodies of different isotypes (Figure 3.1.3). These results showed a functional defect in *Bap1*$^{fl/fl}$ *Cre* mice that is associated with the reduction of splenic B cells.

3.1.3 Cell intrinsic role of BAP1 in B lymphocyte development

"To further validate the cell intrinsic role of BAP1 in B cells, a chimeric mouse model was established. Wild-type CD45.1^{+} marked bone marrow cells were mixed in a 1:1 ratio with bone marrow cells from CD45.2^{+} mice of *Bap1*$^{fl/+}$, *Bap1*$^{fl/+}$*Cre*, or *Bap1*$^{fl/fl}$*Cre* genotypes, and the three mixes transplanted into three independent cohorts of lethally irradiated recipient mice to reconstitute their immune system (Figure 3.1.4A). The recipient mice were analyzed for the relative contribution of CD45.1^{+} and CD45.2^{+} donors to the B cell lineages, by testing the blood at 10 weeks, and the bone marrow and spleen at 17 weeks post-reconstitution. Reduced contribution of the BAP1-deficient *Bap1*$^{fl/fl}$*Cre* donor cells to the B cell lineage was demonstrated in all the tissues (Figure 3.1.4B-E; Figure 3.1.5A), further confirming the cell intrinsic role of BAP1 in B cells" (1).

Figure 3.1.3 Reduction of antigen-specific antibody titers in *Bap1*$^{fl/fl}$ *Cre* mice.



Titers of antigen-specific IgM, IgG1, IgG2c, and IgG3 antibodies in the serum from *Bap1*$^{fl/+}$ (+/+), *Bap1*$^{fl/+}$ *Cre* (Δ/+), and *Bap1*$^{fl/fl}$ *Cre* (Δ/Δ) mice following subcutaneous immunization of R-phycoerythrin (R-PE) in Complete Freund's Adjuvant (14 days "post-prime"), before boost immunization ("pre-boost"), and after boost immunization of R-PE (7 days "post-boost"). Data is from three to six mice per group. Bars represent mean±SEM; *p<0.05, **p<0.01.

Figure 3.1.4 Cell intrinsic role of BAP1 in B cells demonstrated in a chimeric mouse model.

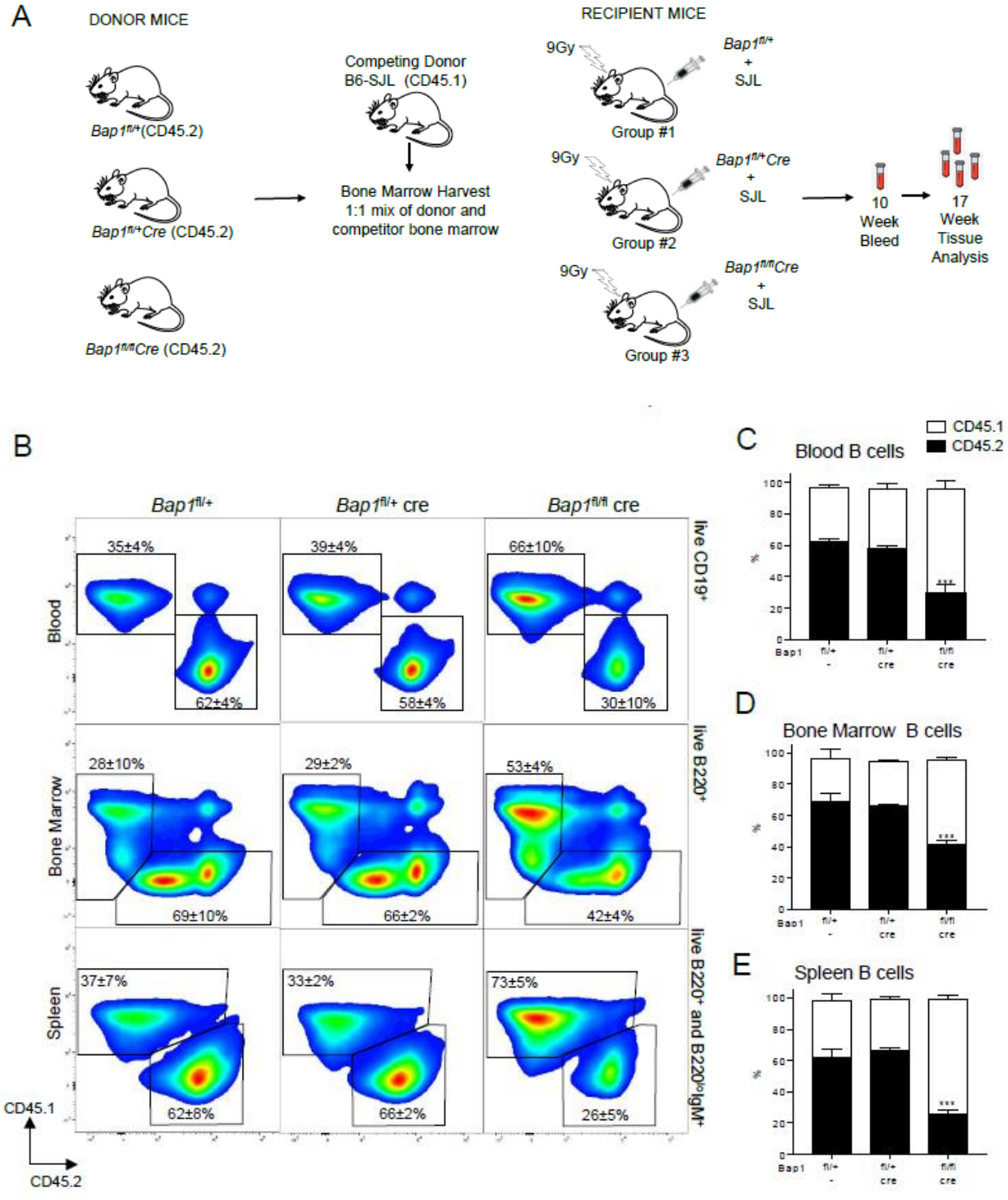


Figure 3.1.4 Cell intrinsic role of BAP1 in B cells demonstrated in a chimeric mouse model.

"**(A)** Schematic diagram of the competitive bone marrow transplantation experiment. Wild-type CD45.1-marked bone marrow cells were mixed in a 1:1 ratio with bone marrow cells from mice of *Bap1*$^{fl/+}$, *Bap1*$^{fl/+}$*Cre*, or *Bap1*$^{fl/fl}$*Cre* genotypes, and the mixes transplanted into three independent cohorts of lethally irradiated recipient mice to reconstitute their immune system. Reduced contribution of *Bap1*$^{fl/fl}$*Cre* donor cells to the B cell lineage is demonstrated with the analysis of blood, bone marrow, and spleen of the recipient mice, at 10 weeks and 17 weeks post-reconstitution, respectively. **(B)** Representative flow cytometry density plots, of the blood, bone marrow, and spleen of the recipient mice, gating on live B cells, as CD19^{+} cells in the blood, B220+ cells in the bone marrow, and B220^{+} or B220^{lo}IgM^{+} cells in the spleen. Gates indicate B cells derived from the CD45.2^{+} test and the CD45.1^{+} competitor bone marrow donors; mean ± S.D. of cell frequency in each gate for all mice within the group is indicated. **(C-E)** Bar charts showing data quantification for **(C)** blood, **(D)** bone marrow, and **(E)** spleen. Bars represent mean ± SEM, data is from n≥4 recipient mice per group, with mice in each recipient group injected with same mix of donor bone marrow cells; statistical comparisons with ANOVA, *** $p<0.001$" (1).

Figure 3.1.5 Supporting data for flow cytometry analyses of *Bap1*$^{fl/fl}$ *Cre* mouse tissues.

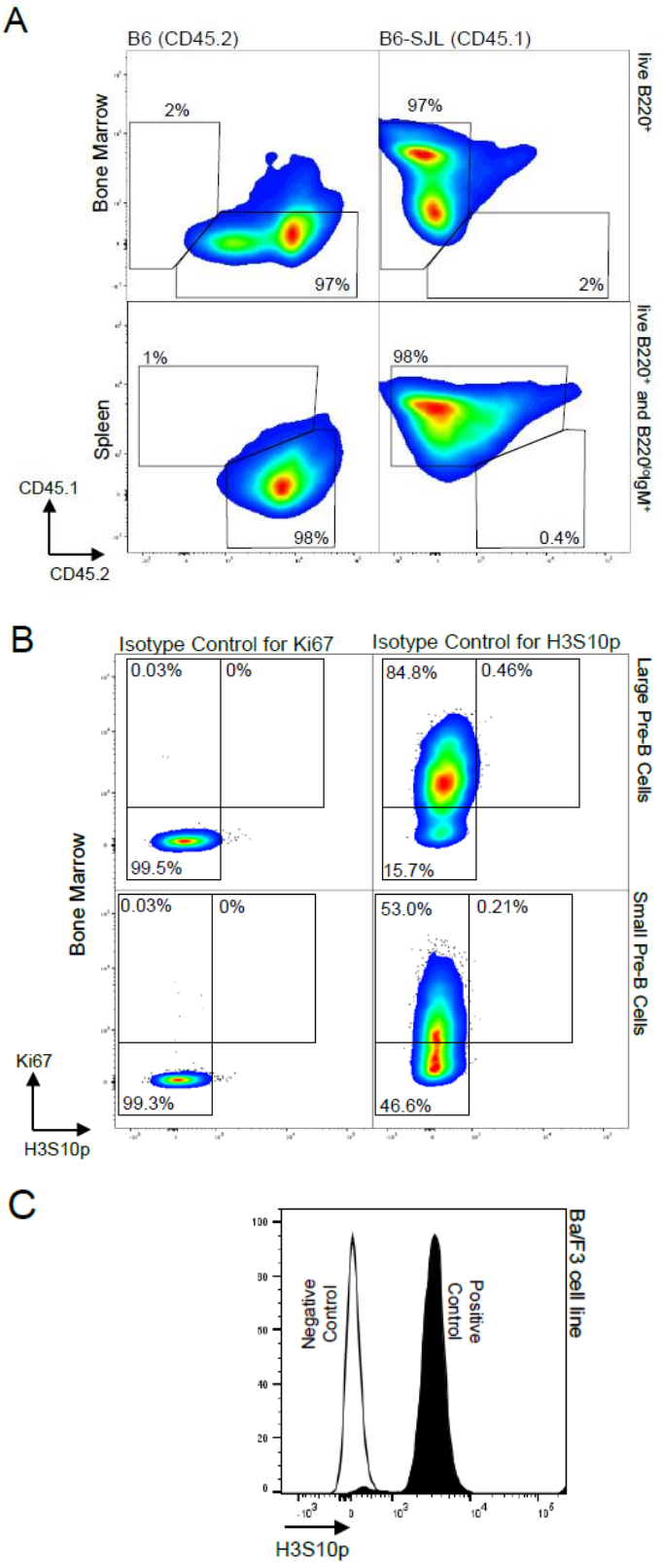


Figure 3.1.5 Supporting data for flow cytometry analyses of *Bap1*$^{fl/fl}$ *Cre* mouse tissues.

"**(A)** Validation of the CD45.2^{+} and CD45.1^{+} B cell population gating. Bone marrow and spleen of control unmanipulated B6 (CD45.2^{+}) and B6-SJL (CD45.1^{+}) mice are analyzed for CD45.2 and CD45.1 marker expression, gating on live B220^{+} cells for the bone marrow, and on live B220^{+} or B220^{lo}IgM^{+} cells for the spleen. Mean cell frequency in the CD45.2^{+} and CD45.1^{+} gates is indicated. **(B)** Validation of cell cycle analyses using Ki67 and histone H3S10p markers - isotype control staining for each marker is shown, gating on live large and live small pre-B cells (B220^{+}IgM^{-}IgD^{-}CD19^{+}CD43^{-}FSC$^{hi/lo}$). Mean cell frequency in the Ki67^{-}H3S10p^{-}, Ki67^{+}H3S10p^{-} and Ki67^{+}H3S10p^{+} gates is indicated. **(C)** Ba/F3 cell line stained for histone H3S10p, as a positive control validation for the staining; histograms are gated on live cells" (1).

3.1.4 Loss of BAP1 impairs B cell development at the pre-B cell stage in the bone marrow

"To analyze the impact of BAP1 loss on B cell development and to pinpoint the specific developmental transitions dependent on BAP1, the bone marrow of *Bap1*$^{fl/fl}$*Cre* and control mice was analyzed by flow cytometry. This demonstrated a significant reduction in the frequency of B cells in the *Bap1*$^{fl/fl}$*Cre* relative to control mice (gated as B220^{+}, Figure 3.1.6A-D). The defect in B cell development in *Bap1*$^{fl/fl}$*Cre* mice was further confirmed with a colony forming units (CFU) assay, demonstrating a reduction in the numbers of pre-B CFUs in *Bap1*$^{fl/fl}$*Cre* bone marrow samples (Figure 3.1.6E). Further characterization of B cell progenitor subpopulations demonstrated a significant depletion in the frequencies and absolute numbers of large pre-B cells and mature B cells, and an expansion of Fraction B pro-B cells, in *Bap1*$^{fl/fl}$*Cre* relative to control bone marrow (Figure 3.1.6F-G). No changes in the abundance of the common lymphoid progenitors, multipotent progenitors, and hematopoietic stem cells were observed (data not shown), further confirming the specificity of the *Cre* transgene and the phenotype to the B cell lineage. Overall, our findings indicate the cell intrinsic role of BAP1 as a regulator of B cell development, and demonstrate that BAP1 loss impacts pre-B cells, as well as transitional and mature B cell subsets" (1).

Figure 3.1.6 BAP1 loss impairs B cell development from the pre-B cell stage in the bone marrow.

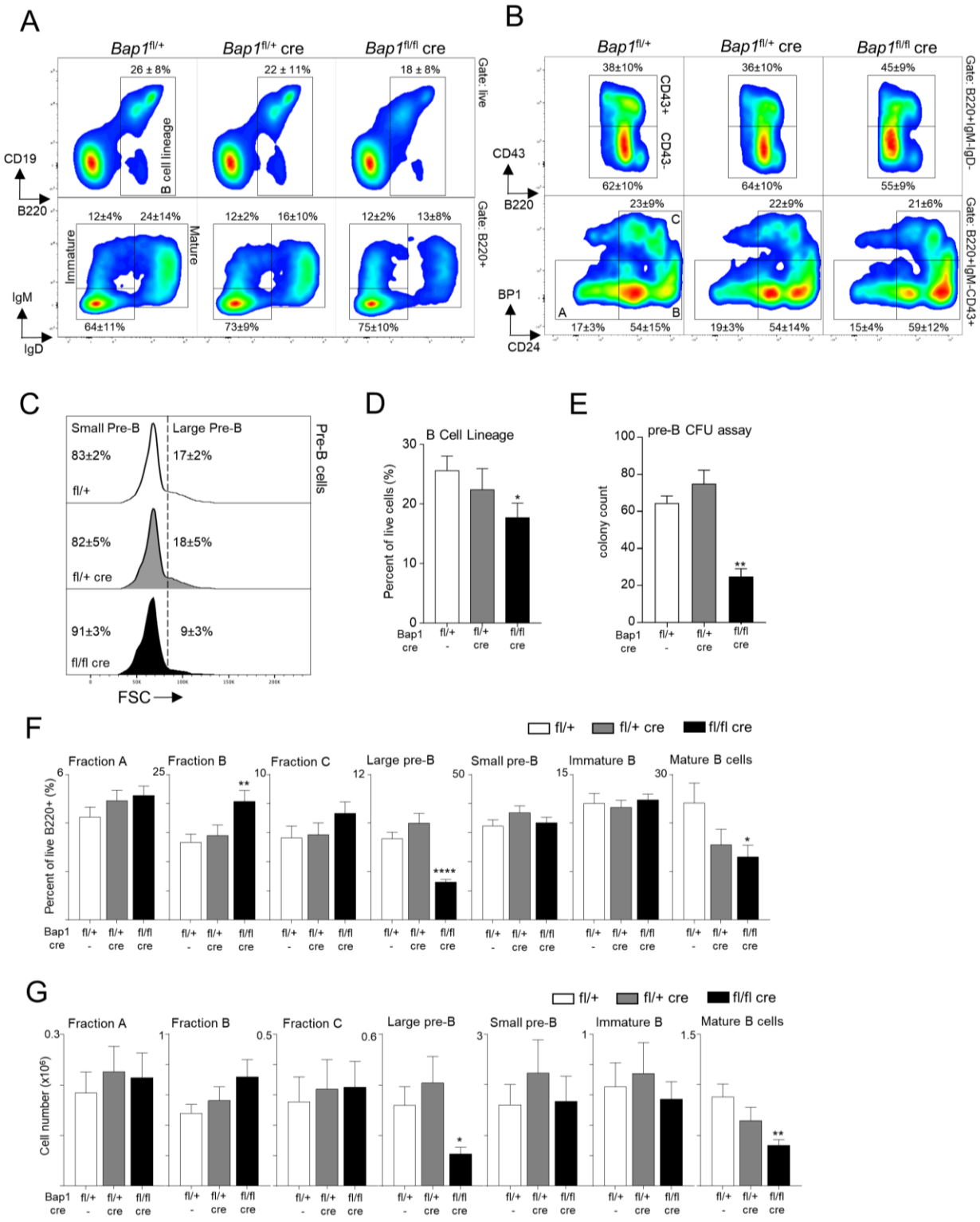


Figure 3.1.6 BAP1 loss impairs B cell development from the pre-B cell stage in the bone marrow. "**(A-B)** Representative flow cytometry plots of the bone marrow of mice of *Bap1*$^{fl/+}$, *Bap1*$^{fl/+}$*Cre*, and *Bap1*$^{fl/fl}$*Cre* genotypes, demonstrating: (**A**, top) gating on B cell lineage cells (B220^{+}); (**A**, bottom) gating on mature B cells (IgM^{+}IgD^{+}) and immature B cells (IgM^{+}IgD^{-}); (**B**, top) gating on pre-B cells (B220^{+} IgM^{-}IgD^{-}CD43^{-}); (**B**, bottom) gating on Hardy Fractions A, B and C cells as B220^{+}IgM^{-}IgD^{-}CD43^{+}, followed by CD24 and BP1 markers. Numbers indicate mean±S.D. of cell frequency in each gate; data is from 10-12 mice per genotype, consolidated across 3 independent experiments. **(C)** Representative flow cytometry histograms gated on B220^{+}IgM^{-}IgD^{-}CD43^{-} pre-B cells, and showing the gating on large and small pre-B cell subsets based on the forward scatter (FSC) of the cells. Numbers indicate mean±S.D. of cell frequency in each gate; data is from 10-12 mice per genotype, consolidated across 3 independent experiments. **(D)** Reduced frequency of B lineage cells (B220^{+}) in the bone marrow of *Bap1*$^{fl/fl}$*Cre* relative to control mice. Data is from 10-12 mice per genotype, consolidated across 3 independent experiments. **(E**) Reduction in pre-B cell colony forming units in the bone marrow of *Bap1*$^{fl/fl}$*Cre* relative to control mice, demonstrating impaired B cell lineage development; colony count is presented per 1x10^{5} mouse bone marrow cells plated per dish; 3 mice were analyzed in total in two independent experiments. **(F-G)** Frequencies and absolute numbers of B cell precursor subpopulations in the bone marrow of *Bap1*$^{fl/fl}$*Cre* and control mice; the cells were gated as live B220^{+} cells followed by IgM^{-}IgD^{-}CD43^{+}CD24^{lo}BP1^{lo} for Fraction A, IgM^{-}IgD^{-}CD43^{+}CD24^{+}BP1^{lo} for Fraction B, IgM^{-}IgD^{-}CD43^{+}CD24^{+}BP1^{+} for Fraction C, IgM^{-}IgD^{-}CD43^{-}FSC^{hi} for large pre-B cells, IgM^{-}IgD^{-}CD43^{-}FSC^{lo} for small pre-B cells, IgM^{+}IgD^{-} for immature B cells, and IgM^{+}IgD^{+} for mature B cells. Bars represent mean ± SEM; flow cytometry data is from 10-12 mice per genotype, consolidated across 3 independent experiments, with cell counts

presented per one tibia and femur. Statistical analyses were performed with ANOVA; *p<0.05, **p<0.01, ***p<0.001" (1).

3.1.5 Impaired cell viability and cell cycle progression in *Bap1*-deficient pre-B cells

"To assess how the loss of BAP1 affects B cell development and physiology, we analyzed the effects of BAP1 loss on cell viability and cell cycle progression throughout the B cell lineage, with flow cytometry. This demonstrated a specific reduction in the viability of large pre-B cells, caused by an increase in the proportion of late apoptotic/necrotic cells within this B cell subset (Figure 3.1.7A-B)" (1).

"To further analyse the effects of BAP1 loss on cell cycle progression, bone marrow samples from *Bap1*$^{fl/fl}$*Cre* and control mice were stained for Ki67 and histone H3S10p, to identify cells in G0 (Ki67^{-}H3S10p^{-}), G1-S-G2 (Ki67^{+}H3S10p^{-}), and M (Ki67^{+}H3S10p^{+}) phases of the cell cycle within each B cell subpopulation. This revealed a significant increase in the fraction of G0 cells and a corresponding decrease in G1-S-G2 cells within the large pre-B cell, small pre-B cell, immature, and mature B cell subpopulations in *Bap1*$^{fl/fl}$*Cre* mice (Figure 3.1.7C-D, Figure 3.1.5B-C), indicating impaired cell proliferation and cell cycle progression. In contrast no differences were observed in the cell cycle state of pre-pro-B and pro-B cells (Figure 3.1.7C). Overall, this indicated that impaired cell survival and proliferation contribute to the depletion of pre-B cells and possibly to the broader defect in B cell lineage development in *Bap1*$^{fl/fl}$*Cre* mice" (1).

Figure 3.1.7 Impact of *Bap1*-deficiency on B cell lineage cell viability and cell cycle progression.

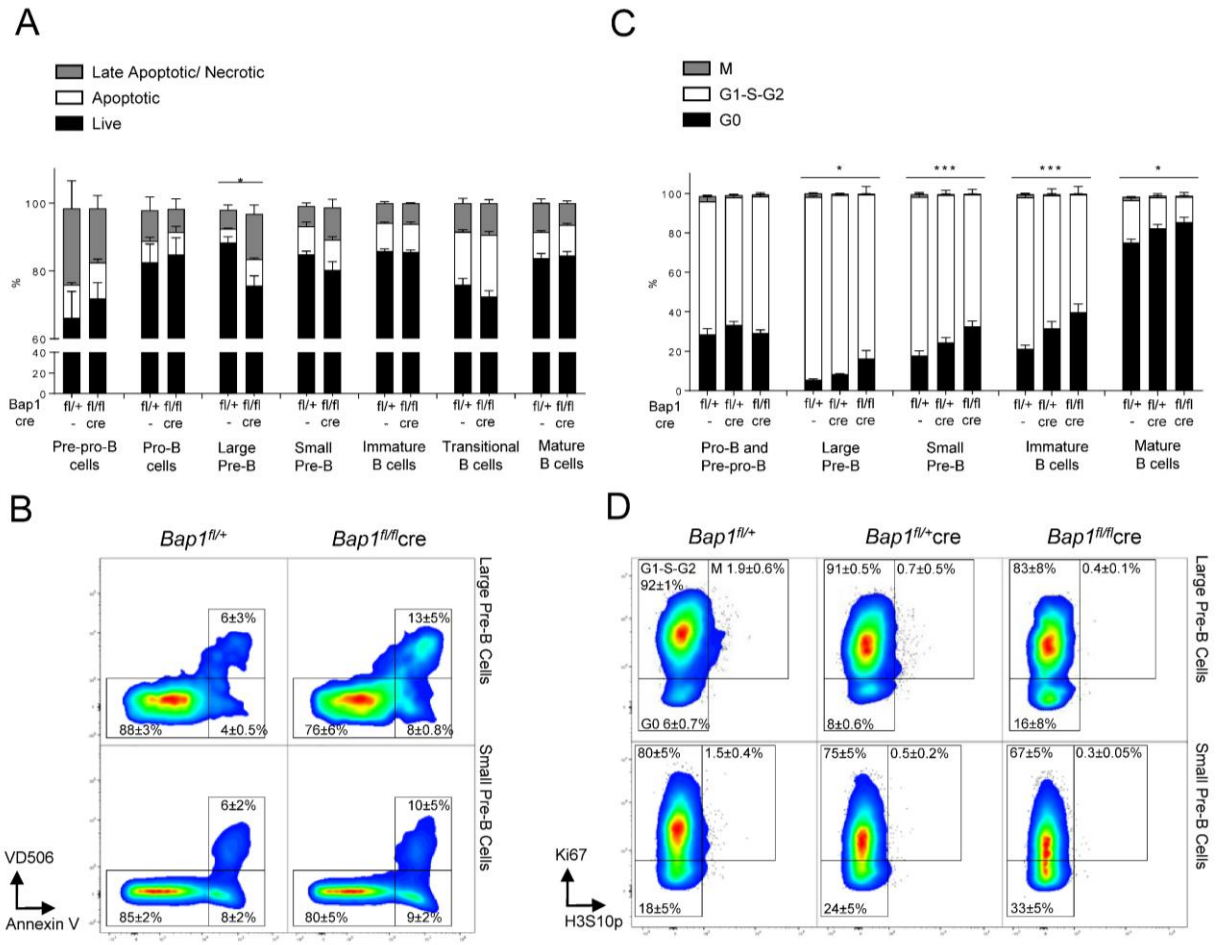


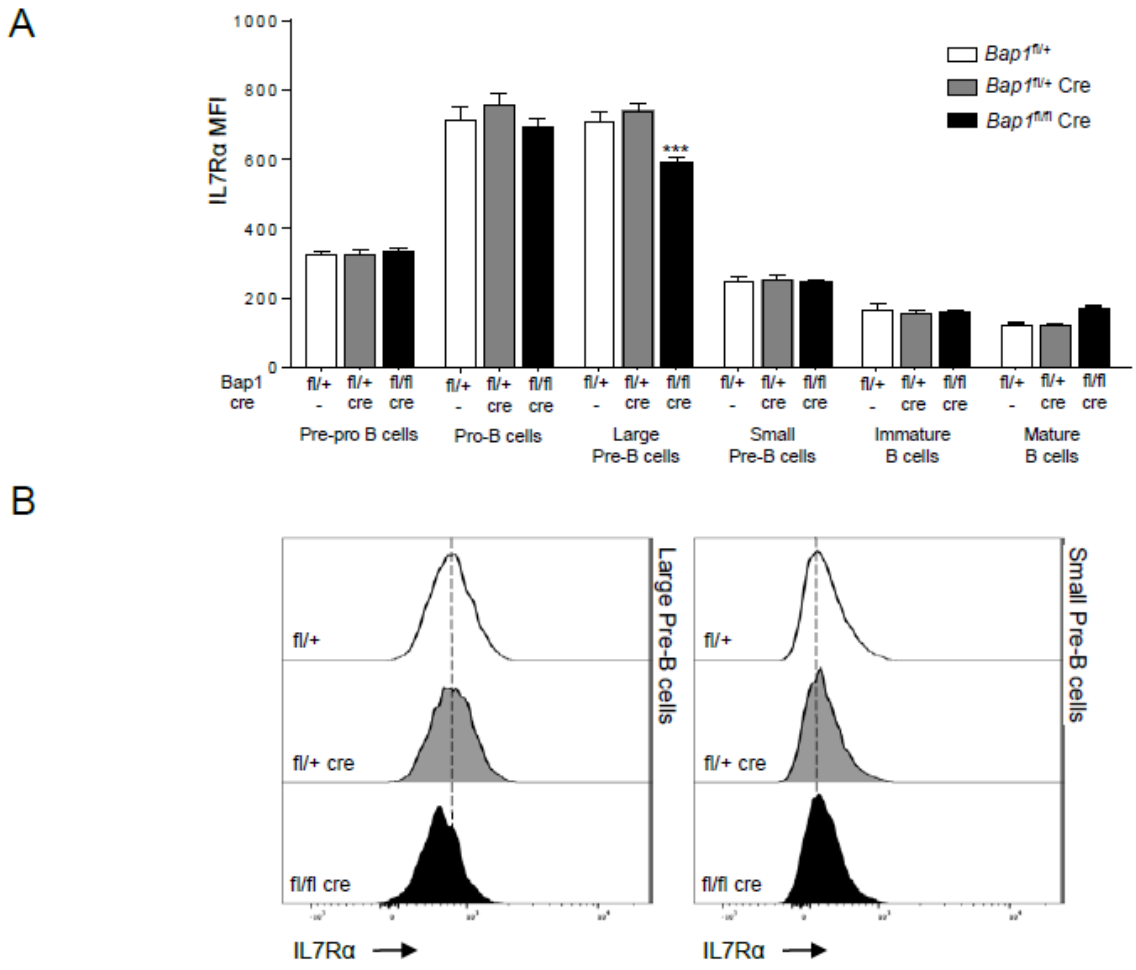
Figure 3.1.7 Impact of *Bap1*-deficiency on B cell lineage cell viability and cell cycle progression. "**(A-B)** Impaired viability of large pre-B cells in *Bap1$^{fl/fl}$Cre* mice. Bone marrow and splenocytes of mice of *Bap1$^{fl/+}$* and *Bap1$^{fl/fl}$Cre* genotypes were stained with Annexin V to identify apoptotic cells and with Viability Dye V506 (eBioscience) to identify necrotic cells, as well as for cell surface markers to identify mature splenic B cells (B220^{+}IgM^{+}IgD^{+}), transitional splenic B cells (B220^{+}IgM^{+}IgD^{-}), immature bone marrow B cells (B220^{+}IgM^{+}IgD^{-}), small pre-B cells (B220^{+}IgM^{-}IgD^{-}CD19^{+}CD43^{-}FSC^{lo}), large pre-B cells (B220^{+}IgM^{-}IgD^{-}CD19^{+}CD43^{-}FSC^{hi}), pro-B cells (B220^{+}IgM^{-}IgD^{-}CD19^{+}CD43^{+}), and pre-pro-B cells (B220^{+}IgM^{-}IgD^{-}CD19^{-}CD43^{+}). **(A)** Frequency of live (AnxV^{-}V506^{-}), early apoptotic (AnxV^{+}V506^{-}), and late apoptotic/necrotic (AnxV^{+}V506^{+}) cells within each B cell subpopulation in *Bap1$^{fl/fl}$Cre* and control mice. Bars represent mean ± SEM; n=4 mice were analyzed per genotype in total over two independent experiments; statistical analysis used ANOVA, * $p<0.05$. **(B)** Representative flow cytometry density plots, gated on the large and small pre-B cells and showing the reduced frequency of live cells and increased frequency of late apoptotic/necrotic cells for the large pre-B cells in *Bap1$^{fl/fl}$Cre* mice. Cell frequency in each gate is indicated as mean±S.D. **(C-D)** Impaired cell-cycle progression in *Bap1$^{fl/fl}$Cre* B cells. Bone marrow of mice of *Bap1$^{fl/+}$*, *Bap1$^{fl/+}$Cre*, and *Bap1$^{fl/fl}$Cre* genotypes was stained for Ki67 and histone H3S10p, to identify G0 (Ki67^{-}H3S10p^{-}), G1-S-G2 (Ki67^{+}H3S10p^{-}), and M phase (Ki67^{+}H3S10p^{+}) cells. **(C)** Frequency of G0, G1-S-G2, and M phase cells within each B cell subpopulation, showing impaired cell cycle progression from the pre-B stage in development onward in *Bap1$^{fl/fl}$Cre* mice. Bars represent mean ± SEM; n=4 mice were analyzed per genotype in total over two independent experiments; statistical analyses were performed using ANOVA; * $p<0.05$, *** $p<0.001$, or not significant if not indicated. **(D)** Representative flow cytometry density plots, gated on large and small pre-B cells and showing

elevated frequency of G0 cells and reduced frequency of G1-S-G2 cells in *Bap1*$^{fl/fl}$*Cre* mice; cell frequency in each gate is indicated as mean ± S.D." (1).

"We further observed a reduction in the expression of IL7Rα on the surface of large pre-B cells, but not other B cell precursor subsets in *Bap1*$^{fl/fl}$*Cre* relative to control mice (Figure 3.1.8). Subsequent RNA-seq analyses of *Bap1*$^{fl/fl}$*Cre* and control pre-B cells (Figure 3.1.10), showed no difference in the expression of *Il7r* gene or surrogate light chain gene *Igll1* in *Bap1*$^{fl/fl}$*Cre* pre-B cells (*Vpreb1-2* did not reach >5CPM expression threshold for quantification). This indicates that the differences in IL7Rα protein expression on pre-B cell surface are likely mediated via indirect mechanisms, and are not a result of direct regulation of its encoding gene by BAP1" (1).

"Given the previously reported roles of BAP1 not only in transcriptional regulation but also in DNA repair (220), we further analyzed the B cell precursor cell populations in the bone marrow of *Bap1*$^{fl/fl}$*Cre* and control mice for the levels of γH2AX as a marker of DNA damage (299) and p53 protein as a marker of DNA damage response activation (300). There were no differences in the levels of γH2AX or p53 between *Bap1*$^{fl/fl}$*Cre* and control cells for any of the B cell precursor cell populations (Figure 3.1.9). This suggested that the role of BAP1 as a regulator of B lymphocyte development is likely independent of its functions in DNA repair and is primarily linked to its role as a transcriptional and epigenetic regulator of gene expression" (1).

Figure 3.1.8 Characterization of IL7Rα expression on B cells in the bone marrow of *Bap1*$^{fl/fl}$ *Cre* mice.



"**(A)** Quantification of IL7Rα expression on B cell lineage cells in the bone marrow of *Bap1*$^{fl/fl}$ *Cre* and control mice. **(B)** Representative histograms showing IL7Rα staining of large and small pre-B cells of *Bap1*$^{fl/fl}$*Cre* and control mice. Cells are gated as live B220^{+}IgM^{-}IgD^{-}CD43^{-} FSC^{hi} or FSC^{lo}, respectively. n=3-4 mice were analyzed per genotype per experiment, and the data is consolidated from 2 experiments for IL7Rα analysis; MFI - mean fluorescence intensity; statistical analyses were performed using ANOVA, *** $p<0.001$, or not significant if not indicated" (1).

Figure 3.1.9 Characterization of γH2AX and p53 levels in B cells in the bone marrow of *Bap1*$^{fl/fl}$ *Cre* mice.

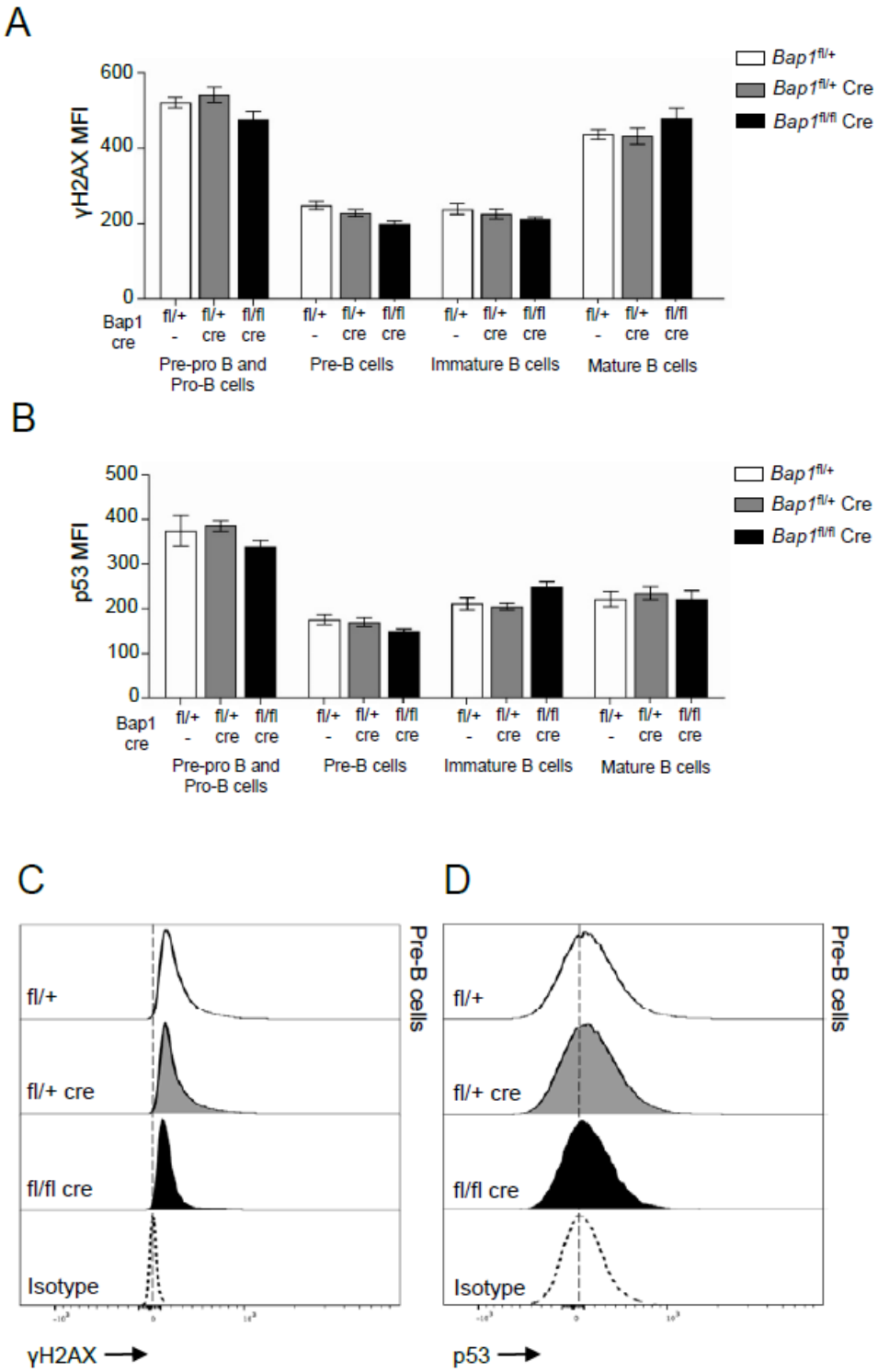


Figure 3.1.9 Characterization of γH2AX and p53 levels in B cells in the bone marrow of *Bap1*$^{fl/fl}$ *Cre* mice. "**(A)** Normal levels of γH2AX DNA damage marker and **(B)** p53 DNA damage response protein in *Bap1*$^{fl/fl}$*Cre* B cells. Cells were gated as B220^{+}IgM^{+}IgD^{+} for mature B cells, B220^{+}IgM^{+}IgD^{-} for immature B cells, B220^{+}IgM^{-}IgD^{-}CD43^{-} for pre-B cells, and B220^{+}IgM^{-}IgD^{-} CD43^{+} for pro-B cells and pre-pro-B cells. Bars represent mean ± SEM; n=4 mice were analyzed per genotype in total over two independent experiments; MFI – mean fluorescence intensity; statistical analyses were performed using ANOVA; not significant if significance is not indicated. **(C-D)** Representative histograms showing γH2AX and p53 staining of pre-B cells of *Bap1*$^{fl/fl}$*Cre* and control genotypes. Negative control histograms from the same cells stained with appropriate isotype control antibodies are also included" (1).

3.1.6 RNA-Seq analysis of BAP1-deficient B cell precursors

"To further understand the functional impact of BAP1 loss on B cell development, RNA-Seq gene expression analysis was conducted on live pre-B and immature B cells, isolated from the bone marrow of *Bap1*$^{fl/fl}$*Cre*, *Bap1*$^{fl/+}$*Cre*, and control *Bap1*$^{fl/+}$ mice through FACS-sorting (Figure 3.1.10A). Dimension reduction analysis of gene expression profiles showed clear segregation of the two cell types (PC1 - 46.5% variance) and the three genotypes (PC2 - 9.3% variance, PC3 - 5.6% variance), and indicated major transcriptional changes in the BAP1-deficient cells (Figure 3.1.10B). To explore the biological functions dysregulated within the transcriptome of BAP1-deficient B cells, a gene set enrichment analysis (GSEA) (298) was performed and highlighted the dysregulation of the transcriptional signatures linked to "cell proliferation", "DNA replication", and "cell division", as well as many other biological processes in *Bap1*$^{fl/fl}$*Cre* cells (Figure 3.1.10C)" (1).

"Differential gene expression analysis across the three *Bap1* genotypes was performed, at fold change ≥1.5 and false discovery rate (FDR) ≤0.01. This identified 916 genes significantly dysregulated in *Bap1*$^{fl/fl}$*Cre* relative to control *Bap1*$^{fl/+}$ cells (Figure 3.1.10D). In contrast, in *Bap1*$^{fl/+}$*Cre* cells the downregulation of *Cd79a* was the only transcriptional change to reach the significance threshold, and is likely due to the direct effect of *Cre* knockin on the *Cd79a* locus (285). This demonstrates the very limited effects of *Cre* or heterozygous *Bap1* loss on the transcriptome, and confirms that the major transcriptional changes in *Bap1*$^{fl/fl}$*Cre* are due to the loss of BAP1 function" (1).

"The dysregulated genes were segregated into Clusters I-VI, based on their upregulation or downregulation status in *Bap1*$^{fl/fl}$*Cre* versus control samples across the two cell types (Figure 3.1.10D). Gene ontology (GO) enrichment analysis was performed for the genes in each cluster, to explore the biological functions dysregulated in the BAP1-deficient B cell precursors. The upregulated transcriptional signatures, represented by Clusters I-III, showed a mild enrichment for GO-terms "immune system process", "cell migration" and "apoptotic process" (Figure 3.1.10E). As an example the upregulated genes included several receptors for chemokines and other messengers (*Cxcr7*, *Cxcr5*, *Cr2*, *IL2rg*, *Il9r*, *S1pr3*, *CD40*, *Ahr*), some pattern recognition receptors (*Tlr1*, *Tlr9*, *Naip5*, *Clec12a*), transcriptional regulators involved in immune cell development and activation (*Irf5*, *Irf7*, *Irf8*, *Nfkb2*), both pro-survival and pro-apoptotic Bcl-family proteins (*Bcl2*, *Bbc3*), and some oncogenes (*Myc*). In contrast, the downregulated transcriptional signature of BAP1-deficient pre-B cells (Cluster V) showed a highly significant enrichment of GO-terms "cell cycle progression" and "DNA replication" (Figure 3.1.10E), indicating the downregulation of genes involved in cell proliferation and cell cycle progression. This is consistent with the depletion of the highly proliferative large pre-B cell subset within the cell population (Figure 3.1.6F-G), but also with the previously reported functions of BAP1 in the regulation of transcriptional programs of cell cycle progression in other cell types (255, 301-304). Overall we hypothesize that a disruption in cell proliferation and cell cycle progression may contribute to the defects in B cell development in BAP1-deficiency" (1).

Figure 3.1.10 RNA-Seq analysis of the transcriptome of *Bap1*-deficient pre-B and immature B cells.

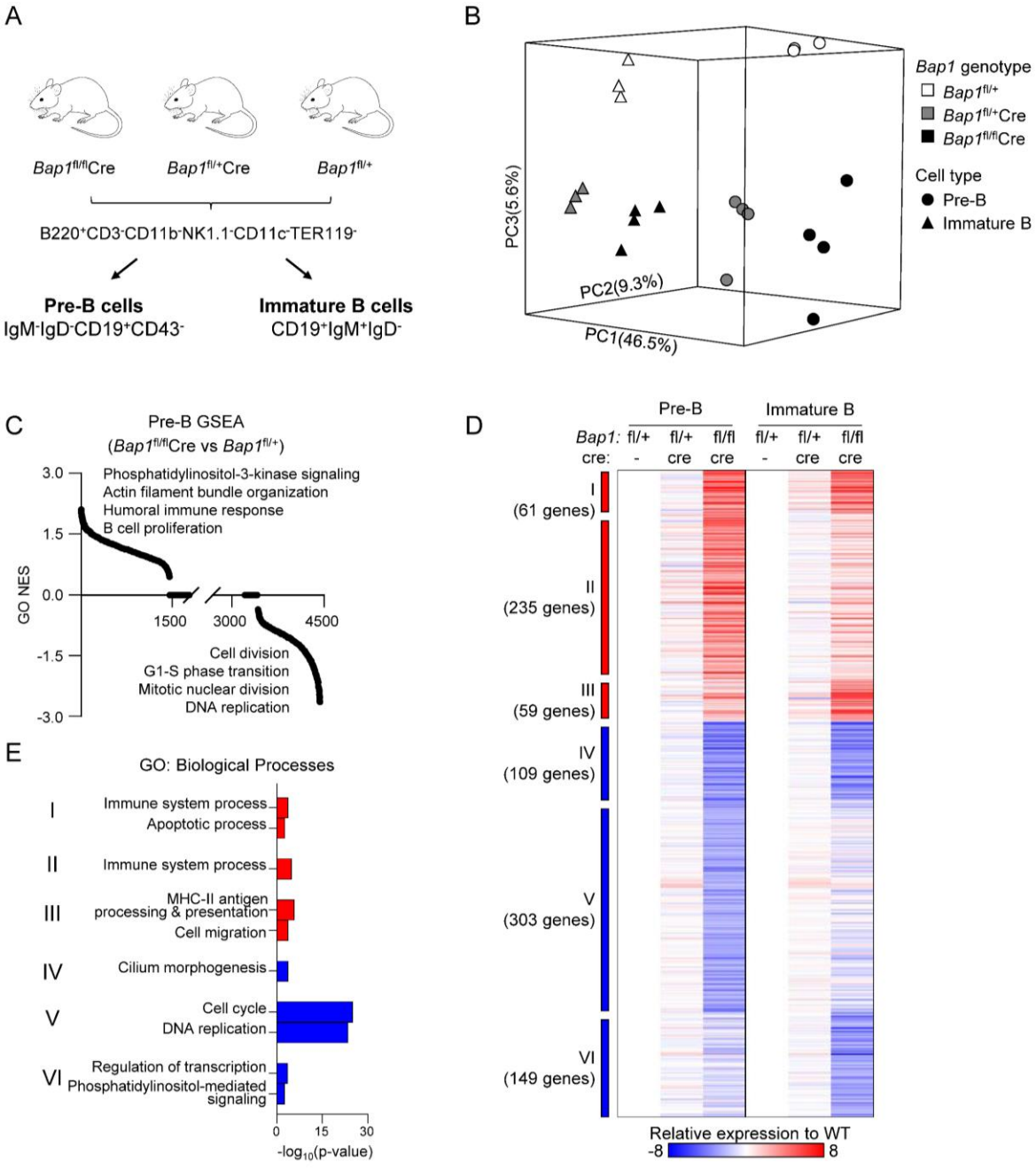


Figure 3.1.10 RNA-Seq analysis of the transcriptome of *Bap1*-deficient pre-B and immature B cells. "Data presented is from 3-4 mice per genotype. **(A)** Schematic representation of the mouse genotypes and cell sorting protocols for the RNA-Seq experiment. **(B)** 3D principal component analysis model demonstrating the gene expression profiles of each RNA-Seq sample: differences between cell types are described by principal component 1 (PC1, 46.5% variability) and differences between genotypes are described by PC2 and PC3 (9.3% and 5.6% variability, respectively). **(C)** Normalized enrichment scores (NES) of 4,436 pre-established biological processes expression signatures used in the gene set enrichment analysis (GSEA). **(D)** Heat map displaying 916 significantly dysregulated genes when comparing *Bap1*$^{fl/fl}$*Cre* and control *Bap1*$^{fl/+}$ cells. The significance threshold is: fold change ≥ 1.5 and False Discovery Rate (FDR) ≤0.01. Relative expressions to the average of pre-B and immature B control *Bap1*$^{fl/+}$ group are used to generate the heat map. Genes are grouped into Clusters I-VI based on their expression pattern across the two cell types and three genotypes. **(E)** Gene ontology (GO) enrichment analysis on the genes from the Clusters I-VI described in (D). The top two enriched biological processes terms per cluster are displayed" (1).

3.2 ROLE OF MYSM1 IN C-MYC-DRIVEN B-CELL LYMPHOMA

3.2.1 Co-localization of MYSM1 and cMYC DNA binding sites at the promoters of *RP* genes

Like BAP1, MYSM1 is a histone deubiquitinase and an important transcriptional regulator of hematopoiesis and B-cell biology (229, 230, 261, 305, 306). Previous studies have elucidated the significance of MYSM1 in normal production of hematopoietic stem cells, T lymphocytes, B lymphocytes, natural killer cells, and dendritic cells; however, the role of MYSM1 as a transcriptional regulator in hematologic malignancies, particularly B-cell lymphoma, remains unexplored (229, 230, 261-263, 268, 305, 306). We recently conducted the first genome-wide analysis of MYSM1-regulated genes and demonstrated that in primary murine hematopoietic stem and progenitor cells MYSM1 promotes the expression of many genes encoding ribosomal proteins and translation factors (269). Interestingly, MYSM1 was previously shown to interact with cMYC in B1a lymphocytes (306). As cMYC is the major transcriptional regulator for the genes encoding the cellular ribosomal and translational machinery, here we investigate the cross-talk between MYSM1 and cMYC in the regulation of these gene-sets and its implications for cMYC driven carcinogenesis.

To compare the location of the genomic binding sites of MYSM1 and cMYC, we consolidated the ChIP-Seq datasets for cMYC and its dimerization partner MAX from multipotent hematopoietic progenitor cells HPC7 (307, 308) with the MYSM1 ChIP-Seq acquired in our recent work in a B cell progenitor cell line Ba/F3 (269). This identified 45 binding sites shared by MYSM1 and cMYC (data by Belle JI and Wang H, Appendix Figure 5.1A-B), all located within 1kb to the nearest gene transcription start site (TSS). Importantly, 28 of these shared binding sites localized near the genes encoding ribosomal proteins and 4 others near the genes encoding

translation factors (data by Belle JI and Wang H, Appendix Figure 5.1C-D). Overall, this suggested a possible cooperation between cMYC and MYSM1 in the transcriptional regulation of genes encoding ribosomal proteins and translation factors.

We have previously validated MYSM1 binding to the promoters of *RP*-genes in Ba/F3 cells by ChIP-qPCR, and also demonstrated a reduction in *RP*-gene expression in *Mysm1*-shRNA knockdown Ba/F3 cells lines (269). To further validate the overlap in the genomic binding sites of MYSM1 and cMYC in Ba/F3 cells, the binding of cMYC at the MYSM1-binding sites of select genes encoding RPs and translation factors was tested and confirmed with ChIP-qPCR (data by Fiore A, Appendix Figure 5.1E). The binding of MYSM1 and cMYC at the shared sites was also confirmed in cells derived from *Eμ-Myc* mouse B cell lymphoma (286, 309), specifically for the *Rpl7* and *Eef1g* gene promoters (data not shown). We further assessed the effect of MYSM1-knockdown on cMYC binding, with ChIP-qPCR analyses comparing *Mysm1*-shRNA knockdown and control Ba/F3 cells (269). We observed no significant effect of MYSM1-knockdown on cMYC binding at select *RP* gene promoters (data not shown). Overall, we demonstrate shared DNA binding of cMYC and MYSM1 at the promoters of genes encoding ribosomal proteins and translation factors. Our data also suggests that MYSM1 maintains *RP*-gene expression not by facilitating cMYC recruitment, but likely by other molecular mechanisms.

3.2.2 Loss of MYSM1 protects against B cell lymphoma onset and progression

As the induction of the transcriptional programs of ribosome biogenesis is critical for cMYC oncogenic activity (310-312), we hypothesized that MYSM1-loss may interfere with cMYC oncogenic functions. This was tested in the *Eμ-Myc* mouse model of B cell lymphoma that overexpresses cMYC under the control of the immunoglobulin heavy chain locus enhancer (286). The *Eμ-Myc* mouse line was crossed to our established *Mysm1*$^{-/-}$ and *Mysm1*$^{fl/fl}$*Cre*^{ERT2} mouse lines, allowing either constitutive or tamoxifen-induced *Mysm1*-deletion (230, 267). Protective effects were seen with *Mysm1*-deletion in both models, as demonstrated by the increased lifespan of *Eμ-Myc Mysm1*$^{-/-}$ and tamoxifen-treated *Eμ-Myc Mysm1*$^{fl/fl}$*Cre*^{ERT2} mice relative to corresponding control *Eμ-Myc* groups (Figure 3.2.1A-B). This indicates that the loss of MYSM1 can inhibit the oncogenic activity of cMYC and delay the onset of fatal lymphoma.

To further establish the protective effect of MYSM1-loss on lymphoma disease progression, we employed an adoptive lymphoma cell transfer model. Lymphoma cells were harvested from *Eμ-Myc Mysm1*$^{fl/fl}$*Cre*^{ERT2} and control *Eμ-Myc Mysm1*$^{fl/fl}$ donor mice, and transferred into independent cohorts of wild type C57BL/6 recipient mice at 10^6 cells per mouse via intravenous injections. The recipient mice were treated either with tamoxifen to induce *Mysm1*-deletion, or with vehicle corn-oil. Thereby, we demonstrated that *Mysm1*-deletion had a striking protective activity, with strong extension in mouse survival and full remission in many of the treated animals (Figure 3.2.1C). This firmly establishes that loss of MYSM1 inhibits the oncogenic activity of cMYC, and protects against B cell lymphoma onset and progression in mouse models.

To understand the mechanisms underlying the protective activity of MYSM1-loss in B cell lymphoma, *Eμ-Myc* tumours were harvested from *Mysm1*$^{-/-}$ and control mice, and lymphoma cells isolated by cell-sorting as live B220^{+} cells, for *ex vivo* analyses with qRT-PCR and intracellular

flow cytometry. We observed a significant downregulation in the expression of genes encoding ribosomal proteins and translation factors in *Eμ-Myc Mysm1*$^{-/-}$ relative to control *Eμ-Myc* lymphoma cells (Figure 3.2.1D). A reduction in the translation factor eEF1G in *Eμ-Myc Mysm1*$^{-/-}$ lymphoma cells was further validated by flow cytometry at the protein level (Figure 3.2.1E). This was associated with a reduction in the overall protein synthesis rate in *Eμ-Myc Mysm1*$^{-/-}$ relative to control *Eμ-Myc* lymphoma cells, and an increase in the levels of the p53 tumour suppressor protein (Figure 3.2.1F-G). Importantly, previous studies in the *Eμ-Myc* mouse model have shown that ribosomal dysfunction can restrain cMYC oncogenic activity via both a reduction in cellular protein synthesis (313) and the activation of p53 (314). In future work, it will be important to address the relative contribution of these pathways to the protective effects of *Mysm1*-deficiency in *Eμ-Myc* B cell lymphoma, and this will provide insights into the possible effects of acquired p53 mutations on this protective activity. Overall, we establish that the loss of MYSM1 protects against B cell lymphoma onset and progression in the *Eμ-Myc* mouse model via a reduction in the expression of genes encoding the cellular ribosomal and translational machinery.

Figure 3.2.1 Loss of MYSM1 protects against B cell lymphoma onset and progression in mouse models, via the reduction in expression of the genes encoding ribosomal and protein translation machinery.

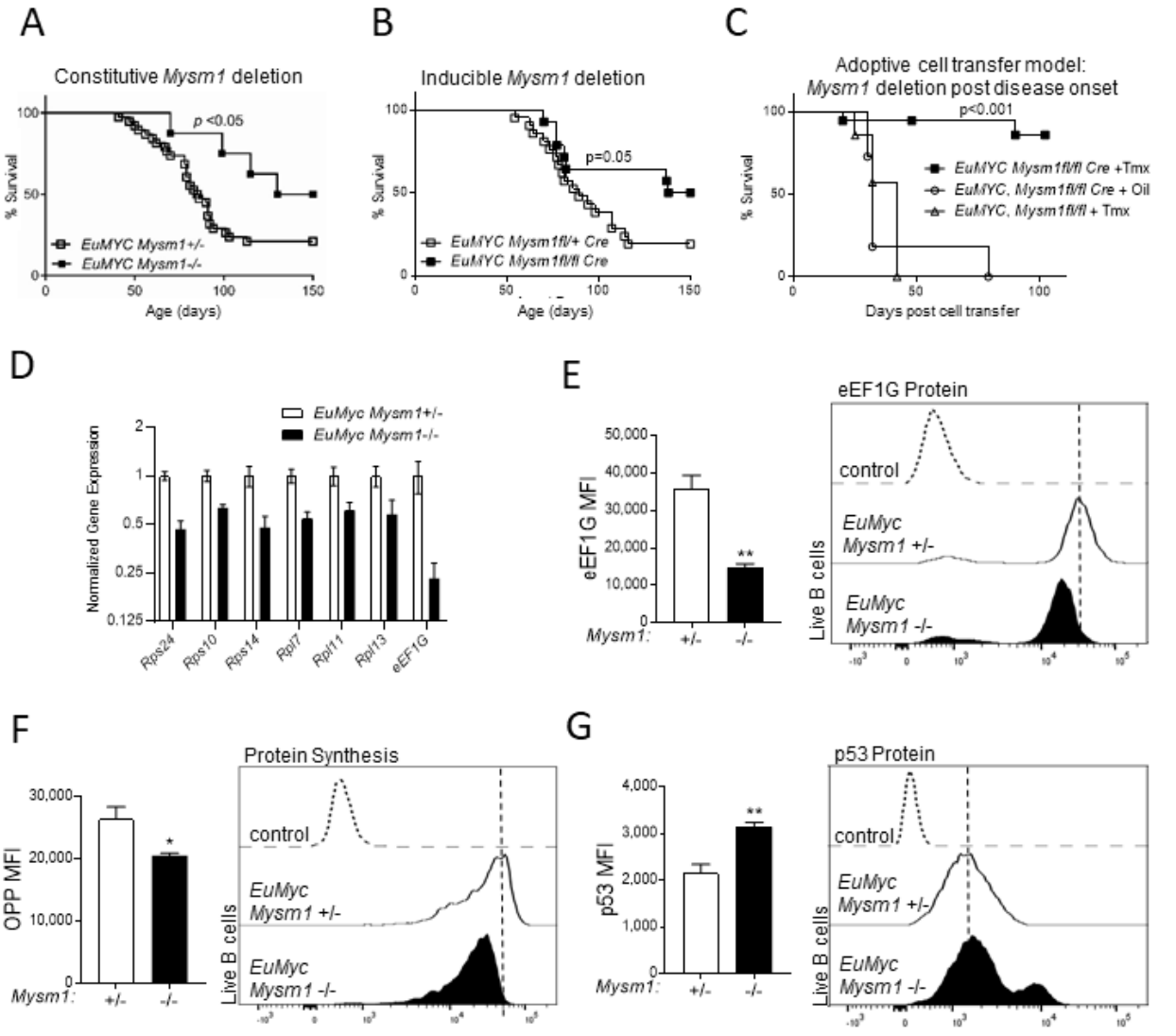


Figure 3.2.1 Loss of MYSM1 protects against B cell lymphoma onset and progression in mouse models, via the reduction in expression of the genes encoding ribosomal and protein translation machinery. (A) Survival of *Eμ-Myc Mysm1*$^{-/-}$ (n=8) relative to control *Eμ-Myc Mysm1*$^{+/-}$ littermates (n=38). **(B)** Survival of tamoxifen-treated *Eμ-Myc Mysm1*$^{fl/fl}$ *Cre*^{ERT2} mice (n=14), relative to tamoxifen-treated *Eμ-Myc Mysm1*$^{fl/+}$ *Cre*^{ERT2} control littermates (n=21). Note that *Mysm1*$^{+/-}$ and *Mysm1*$^{fl/+}$ were used as controls, as they were bred as littermates, age and sex matched, and maintained in the same cages as mice of the main experimental groups; mice lacking one *Mysm1* allele are known to be phenotypically equivalent to wild type, according to previous studies (230, 269). **(C)** Survival of wild type recipient mice after adoptive transfer of 10^6 *Eμ-Myc Mysm1*$^{fl/fl}$ *Cre*^{ERT2} lymphoma cells followed by tamoxifen (Tmx) treatment (n=18), relative to control recipient mice administered with the same number of *Cre*^{ERT2}-negative lymphoma cells followed by Tmx-treatment (n=7), and relative to control recipients receiving cells of the same genotype followed by vehicle corn oil (n=11). *p*-values are calculated using Log-rank (Mantel-Cox) test. **(D-I)** Characterization of *Eμ-Myc* primary lymphoma cells from *Eμ-Myc Mysm1*$^{-/-}$ and control *Eμ-Myc Mysm1*$^{+/-}$ mice. **(D)** Reduced expression of the genes encoding ribosomal proteins and the translation factor eEF1G in *Mysm1*-deficient relative to control *Eμ-Myc* lymphoma cells, measured by qRT-PCR and normalized to *Hprt* and to the average expression in the *Eμ-Myc Mysm1*$^{+/-}$ control group. Live B220$^+$ cells were FACS-sorted from tumours for RNA isolation and qRT-PCR analyses. **(E)** Reduced levels of eEF1G translation initiation factor in the *Mysm1*-deficient relative to control *Eμ-Myc* cells measured by intracellular flow cytometry. **(F)** Protein synthesis rates measured using OPP-incorporation method and flow cytometry, showing a reduction in *Mysm1*-deficient relative to control *Eμ-Myc* lymphoma cells. **(G)** Elevated levels of p53 protein in *Mysm1*-deficient relative to control *Eμ-Myc* lymphoma cells, measured with

intracellular flow cytometry. In panels **(E-G)** mean fluorescence intensity (MFI) of live $B220^+$ lymphoma cells is plotted for each genotype and parameter studied, and representative flow cytometry histograms showing OPP incorporation, eEF1G levels, and p53 levels in live $B220^+$ lymphoma cells of each genotype are provided, with the control samples representing non-specific background staining estimated with isotype control antibodies. Statistical analyses using Student's *t*-test, * $p<0.05$, ** $p<0.01$.

3.3 ROLE OF USP44 IN HSC AND B LYMPHOCYTE FUNCTIONS

3.3.1 *Usp44* is dispensable for HSC function and radioresistance

Similar to BAP1 and MYSM1, USP44 is also a histone deubiquitinase; however, its roles in hematopoiesis and B-cell function are unknown (178, 198, 200). "Given the reported roles of USP44 in cell proliferation, differentiation, DNA repair, and carcinogenesis, and the expression of *Usp44* gene in hematopoietic and immune cells, we hypothesized that USP44 may be essential for these physiologic systems (280). We therefore conducted the first in-depth characterization of hematopoietic and immune functions of *Usp44*-knockout mice" (2).

"Usp44-deficient mice were viable, born in normal numbers, and had no obvious dysmorphology (Figure 3.3.1 and Table 3.3.1), consistent with previous reports (273). Hematopoietic and lymphoid organs were normal in size and cellularity (Figure 3.3.2). Mouse bone marrow was analyzed for the numbers of hematopoietic stem and progenitor cells, gated as $Lin^{-}cKit^{+}Sca1^{+}$ and subdivided into HSCs and multipotent progenitors (MPPs) based on CD150, CD48, CD34, and FLT3 marker expression. No differences were seen in the frequencies or absolute numbers of HSCs and MPPs between genotypes (Figures 3.3.3A–C). Serial bone marrow transplantations were performed as a test of HSC function, comparing the capacity of $Usp44^{+/+}$ and $Usp44^{-/-}$ donor cells to engraft hematopoiesis in lethally irradiated recipients in direct competition with $CD45.1^{+}$ allotype-marked cells. No defects in $Usp44^{-/-}$ HSC function were observed, with normal $Usp44^{-/-}$ contribution to the myeloid cell lineage (Figure 3.3.3D) and to stem cell populations (Figures 3.3.3E–F) in both primary and secondary recipients. As a further measure of stress hematopoiesis, the mice were challenged with whole-body irradiation (6–7 Gy) and no differences in survival were observed between the $Usp44^{+/+}$ and $Usp44^{-/-}$ genotypes (Figure

3.3.3G). We conclude that USP44 is dispensable for normal maintenance of HSC numbers and function under homeostatic conditions and after serial transplantation or irradiation" (2).

Figure 3.3.1 Validation of *Usp44*$^{-/-}$ mouse genotype.

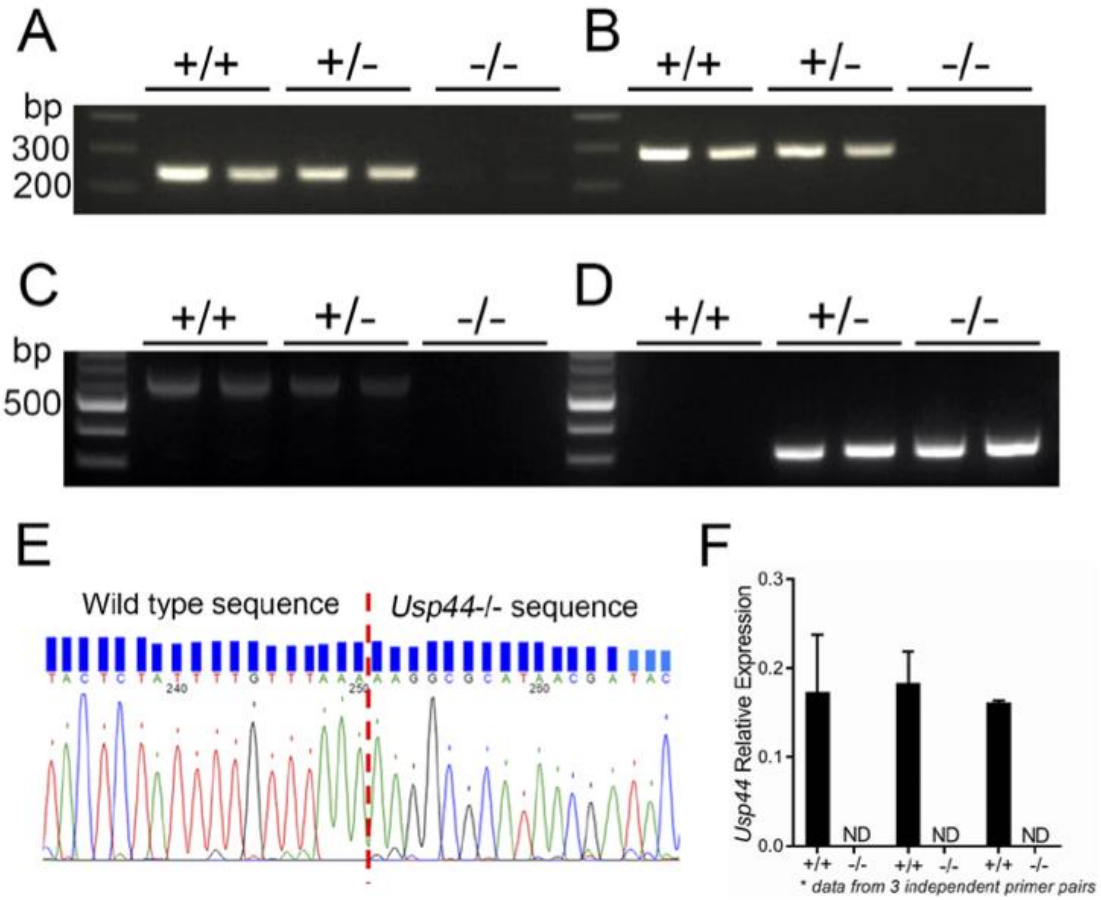


Figure 3.3.1 Validation of *Usp44*$^{-/-}$ mouse genotype. "**(A-D)** Genomic PCR based validation: representative results from *Usp44*$^{+/+}$, *Usp44*$^{+/-}$, and *Usp44*$^{-/-}$ mouse samples are provided; primer sequences are available in Table 3.3.2. **(A-B)** PCRs validating loss of *Usp44* exon *ENSMUSE00001396196* in the genomic DNA of *Usp44*$^{-/-}$ mice; the exon encodes first 475 out of 711 amino acids of USP44 protein including the catalytic site. **(C)** PCR validating the disruption of the intron upstream of *Usp44* exon *ENSMUSE00001396196* with the "gene-trap cassette" in *Usp44*$^{-/-}$ mice. **(D)** Mutant allele specific PCR validating the presence of the "gene-trap cassette" at the intended genomic location within the *Usp44* locus in *Usp44*$^{+/-}$ and *Usp44*$^{-/-}$ mice. **(E)** Sequencing of the mutant allele specific PCR product, validating the disruption of the *Usp44* locus with the "gene-trap cassette" at the intended genomic location. **(F)** qRT-PCR analysis validating loss of *Usp44* expression in *Usp44*$^{-/-}$ B cells; three independent primer pairs used in the analysis are provided in Table 3.3.3; expression normalized to *Hprt* housekeeping control" (2).

Table 3.3.1 *Usp44*$^{-/-}$ mice are born at normal Mendelian frequency.

"Comparison of the genotypes of litters obtained from the mating of two heterozygous animals, against the expected Mendelian frequency" (2).

$\chi^2 = 0.671$, $p = 0.72$.

Genotype	Mice	+/+	+/-	-/-
Observed	#	22	45	18
	%	26	53	21
Expected	#	21	43	21
	%	25	50	25

Figure 3.3.2 Cellularity of hematopoietic and lymphoid organs of *Usp44*$^{-/-}$ mice.

"Bars represent mean ± SEM; data are from 4-5 mice per group. Statistical analysis uses ANOVA with Bonferroni post-hoc test; no significant differences were found; NS - non-significant" (2).

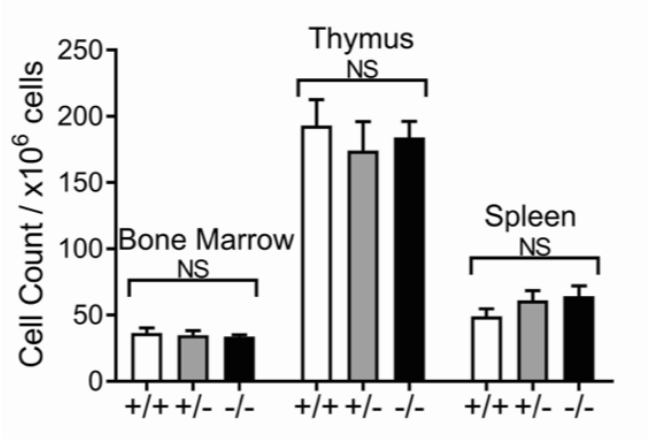


Table 3.3.2 Primers for the validation of *Usp44*$^{-/-}$ mouse genotype.

"Regrettably, no specific antibodies against USP44 are available, and this has precluded the validation for loss of USP44 protein in our samples. The lack of specific anti-USP44 antibodies is extensively discussed in Zhang Y et al., *J Clin Invest*. 2012;122(12):4362-4374 (273). In that study, the authors screened 7 published and commercially available anti-USP44 antibodies, included F-15, N-14, and S-14 (Santa Cruz Biotechnology), GTX87933 (GeneTex), SAB4503322 and HPA026543 (Sigma Aldrich), and 15521-1-AP (Proteintech). They also raised polyclonal antisera using 6 distinct immunogens in a total of 10 rabbits. None of the antibodies were found to be specific for USP44. Additionally, in our own work rabbit polyclonal anti-USP44 (PA539516, Life Technologies) was tested and similarly found to be non-specific. Therefore, we provide extensive genomic-PCR, qRT-PCR, and sequencing based analysis validating the loss of exon *ENSMUSE00001396196* of the *Usp44* coding transcript *ENSMUST00000216224.1*, corresponding to *NM_001206851*. The primers for genomic-PCR are listed in the table below and the gels provided in Figure 3.3.1A-D. The deleted exon encodes the first 475 out of 711 amino acids of USP44 protein, including the catalytic site. Information about the structure of the *Usp44*-tm1b allele is available at http://www.idcc.org/kb/entry/89/ and https://www.infrafrontier.eu/search" (2).

PCR Product Detected		Primer Fw	Primer Rv	PCR Product Size / BP	
				Wild type	*Usp44*-tm1b
1	Wild Type PCR – exon *ENSMUSE00001396196*	CTACTTCGGAAGACAGGACTTT	TGACGGCTGAGTGCTTATAG	240	-
2	Wild Type PCR - exon *ENSMUSE00001396196*	CGCTACCTACTTCGGAAGAC	CTCTTGCCCTTCGTTCATCT	284	-
3	Wild Type PCR - intron upstream of exon *ENSMUSE00001396196*	GCACCCATATACACCGAATGG	TTTCACTGTTCTCCCCGTCC	592	-
4	Mutant/Knockout Allele PCR	GCACCCATATACACCGAATGG	TCGTGGTATCGTTATGCGCC	-	252

Table 3.3.3 Primers used for qRT-PCR analyses of *Usp44* and *Usp49* gene expression.

"Three primer pairs were used per gene and gave equivalent results; all primer sequences were designed in house except for *Usp49*_Fw/Rv3 where the sequence was obtained from www.origene.com" (2).

Primer Name	**Sequence**
*Usp44*_Fw1	CCA CTT CCC AAA GGA GAC TTA T
*Usp44*_Rv1	CAC TGG TAT CTC TCT GGA AAC TC
*Usp44*_Fw2	CCA CTT CCC AAA GGA GAC TTA TT
*Usp44*_Rv2	GAC AAG TCC CAG AAG GAT TCT ATG
*Usp44*_Fw3	GCA AGT TCT GAA TGT GGT GAA TAA C
*Usp44*_Rv3	GCA CTG GTA TCT CTC TGG AAA C
*Usp49*_Fw1	GTC CCT TTG AAT CAG ACA GAG T
*Usp49*_Rv1	TCC TAG CTT CAC TCA GAA CAA G
*Usp49*_Fw2	GAA TTC CCT GAA CGA TAC CAC T
*Usp49*_Rv2	TAA CTG CTT CCT AGC TTC ACT C
*Usp49*_Fw3	GGA GAA TCT ACG CTT GTG ACC AG
*Usp49*_Rv3	GGA GGA CCT GAG GTA GTC TGT A

Figure 3.3.3 Loss of *Usp44* does not impair HSC function.

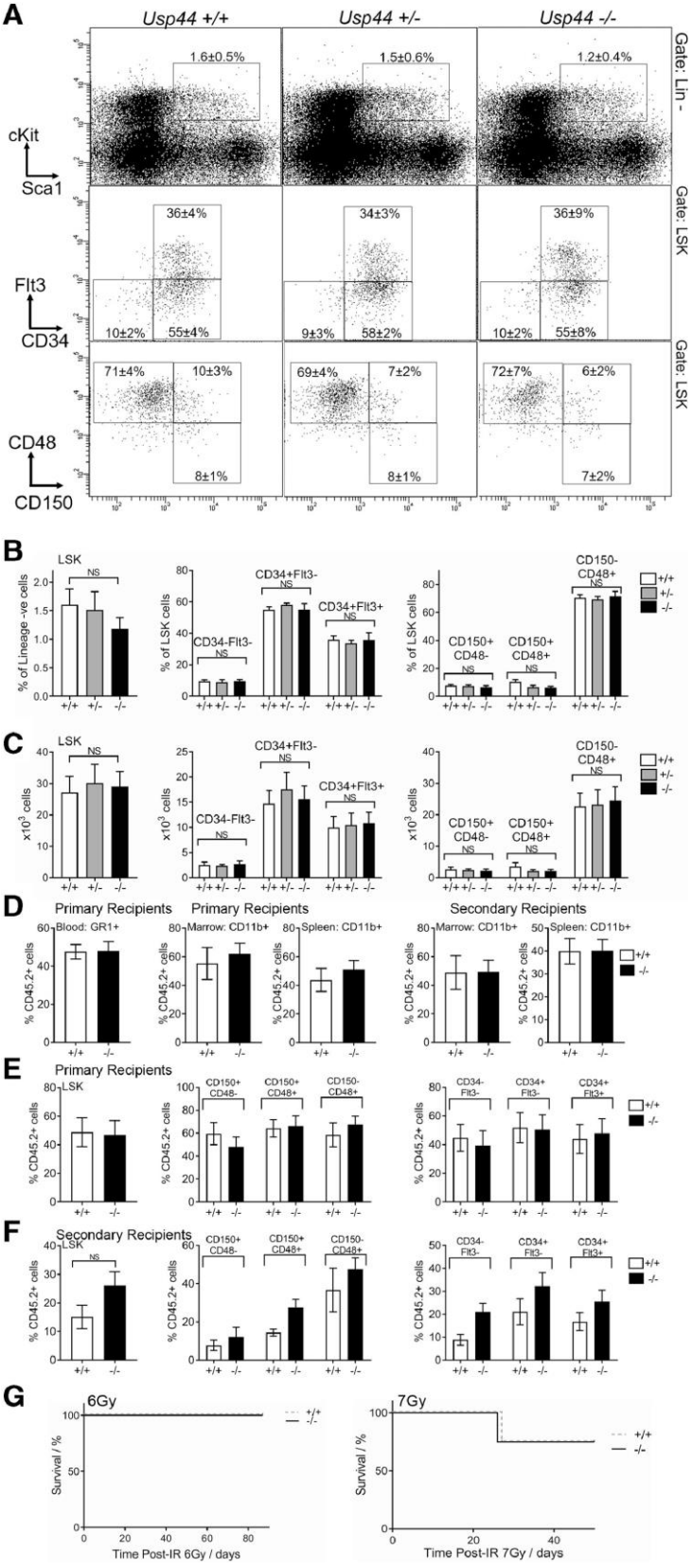


Figure 3.3.3 Loss of *Usp44* does not impair HSC function.

"**(A–C)** Flow cytometry analysis of HSCs and MPPs in the bone marrow of *Usp44*$^{+/+}$, *Usp44*$^{+/-}$, and *Usp44*$^{-/-}$ mice. **(A)** Representative flow cytometry dot plots. Hematopoietic stem and progenitor cells were gated as Lin^{-}cKit^{+}Sca1^{+} (LSK, top panel), followed by either CD34/Flt3 or CD150/CD48 to separate the subpopulations. HSCs were gated as LSK CD34^{-}Flt3^{-} (middle panel) or LSK CD150^{+}CD48^{-} (bottom panel). **(B,C)** Numerical comparisons of the **(B)** frequencies and **(C)** absolute numbers of LSKs, MPPs, and HSCs in the bone marrow of the mice. **(D–F)** Analysis of *Usp44*$^{-/-}$ HSC function using serial bone marrow transplantation. For the primary transplantation, two cohorts of lethally irradiated recipient mice were injected intravenously with a 1:1 mixture of CD45.1^{+} and CD45.2^{+} donor bone marrow cells, with the CD45.2^{+} cells derived either from the control *Usp44*$^{+/+}$ or the test *Usp44*$^{-/-}$ mice. For secondary transplantations, bone marrow cells harvested from the primary recipients were injected into secondary cohorts of lethally irradiated recipients. All recipients were analyzed at >20 weeks after reconstitution, comparing the contribution of control *Usp44*$^{+/+}$ CD45.2^{+} and test *Usp44*$^{-/-}$ CD45.2^{+} HSCs with the different hematopoietic cell types and lineages. **(G)** Mouse survival following total body irradiation at the dose of 6 Gy (left panel) or 7 Gy (right panel), comparing the radioresistance of *Usp44*$^{+/+}$ and *Usp44*$^{-/-}$ mouse bone marrow. Data are from four or five mice per group per experiment and were reproduced in two independent experiments, with the exception of secondary bone marrow transplantation, which was carried out in a single experiment. Bars represent mean ± SEM; statistical analysis was done using the Student *t* test for two groups, ANOVA with Bonferroni post hoc test for multiple comparisons and Kaplan–Meier regression analysis and log–rank test for survival data; no significant differences were found. NS=nonsignificant" (2).

3.3.2 *Usp44* is dispensable for lymphocyte development

"Detailed flow cytometry analysis of hematopoietic and lymphoid organs of *Usp44*$^{+/+}$, *Usp44*$^{+/-}$, and *Usp44*$^{-/-}$ mice was carried out to assess B- and T-cell development. *Usp44*$^{-/-}$ mice had normal frequencies and absolute numbers of all the major thymocyte populations, as well as mature CD4 T cells, CD8 T cells, and natural killer (NK) cells in peripheral lymphoid organs (Figures 3.3.4A and C–E). B-cell development was also found to be normal (Figures 3.3.4B, F, and G). Lymphocyte development also progressed normally following transplantation of *Usp44*$^{-/-}$ bone marrow into lethally irradiated recipients in direct competition against wild-type CD45.1^{+} cells, with normal reconstitution of *Usp44*$^{-/-}$ T cells, B cells, and NK cells in both primary and secondary recipients (Figure 3.3.4H)" (2).

Figure 3.3.4 Loss of USP44 does not impair lymphocyte development.

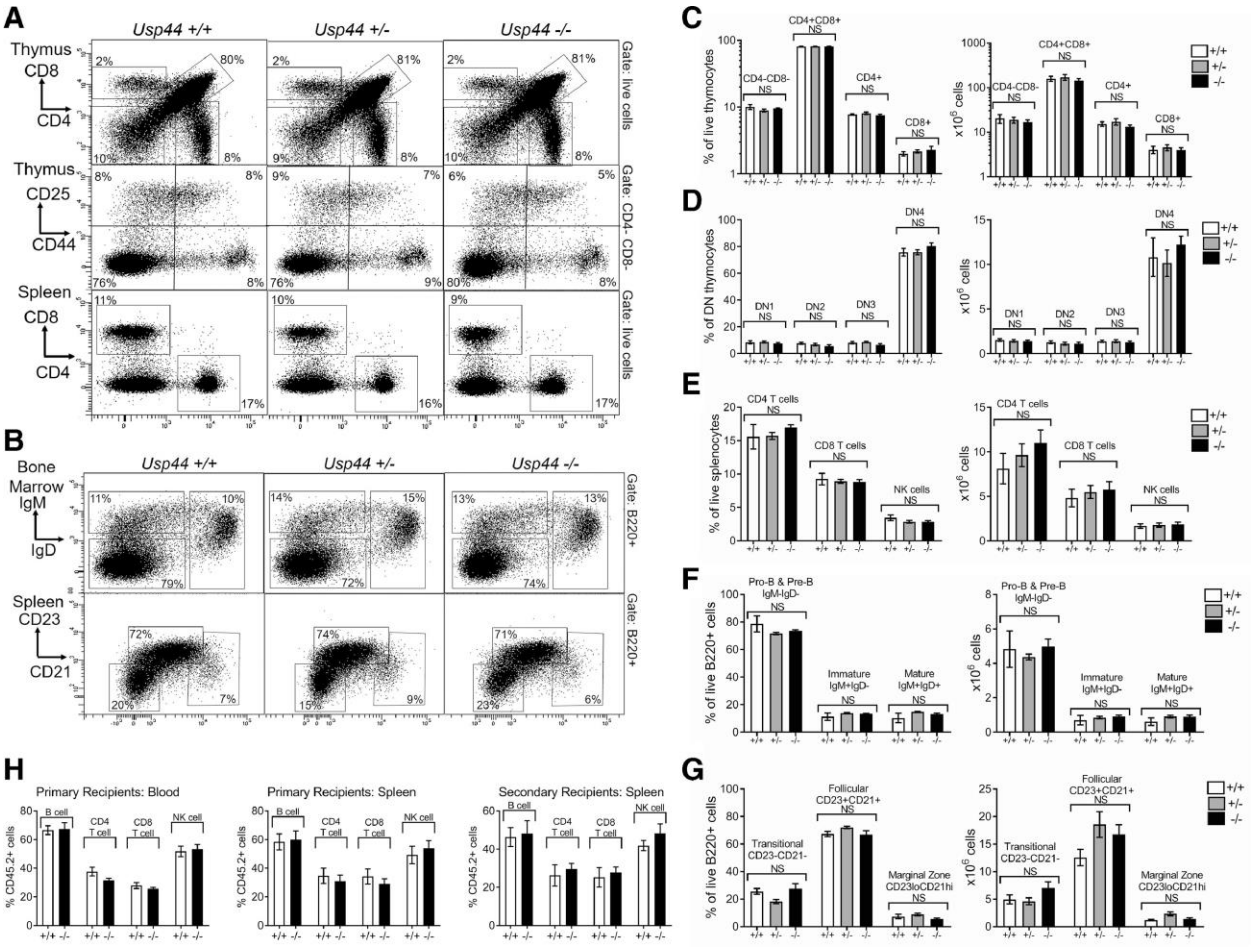


Figure 3.3.4 Loss of USP44 does not impair lymphocyte development. "**(A–G)** Flow cytometry analysis of T- and B-cell development in the thymus, bone marrow, and spleen of *Usp44*$^{+/+}$, *Usp44*$^{+/-}$, and *Usp44*$^{-/-}$ mice. **(A)** Representative flow cytometry dot plots of the thymus and spleen showing CD4^{-}CD8^{-} double-negative (DN), CD4^{+}CD8^{+} double-positive (DP), and CD4^{+}CD8^{-} and CD4^{-}CD8^{+} single-positive cells, as well as CD4^{-}CD8^{-}CD44^{+}CD25^{-} DN1, CD4^{-}CD8^{-} CD44^{+}CD25^{+} DN2, CD4^{-}CD8^{-}CD44^{-}CD25^{+} DN3, and CD4^{-}CD8^{-}CD44^{-}CD25^{-} DN4 cells. **(B)** Representative flow cytometry dot plots of the bone marrow and spleen showing B220^{+}IgM^{-}IgD^{-} pro- and pre-B cells, B220^{+}IgM^{+}IgD^{-} immature B cells, and B220^{+}IgM^{+}IgD^{+} mature B cells in the bone marrow (top panel), as well as B220^{+}CD23^{-}CD21^{-} transitional, B220^{+}CD23^{+}CD21^{+} follicular, and B220^{+}CD23^{lo}CD21^{hi} marginal zone B cells in the spleen**. (C–G)** Numerical comparisons of the frequencies and absolute numbers of **(C,D)** thymocytes, **(E)** splenic T cells and NK cells, **(F)** bone marrow B-cell precursors, and **(G)** splenic B-cell populations in *Usp44*$^{+/+}$, *Usp44*$^{+/-}$, and *Usp44*$^{-/-}$ mice. **(H)** Reconstitution of B cells, T cells, and NK cells in the blood and spleen by donor *Usp44*$^{+/+}$ or *Usp44*$^{-/-}$ HSCs following competitive bone marrow transplantation. For primary transplantation, two cohorts of lethally irradiated recipient mice were injected intravenously with a 1:1 mixture of CD45.1^{+} and CD45.2^{+} donor bone marrow cells, with the CD45.2^{+} cells derived either from the control *Usp44*$^{+/+}$ or the test *Usp44*$^{-/-}$ mice. For secondary transplantation, bone marrow harvested from the primary recipients was injected into secondary cohorts of lethally irradiated recipients. All recipients were analyzed at >20 weeks after reconstitution, comparing the contribution of control *Usp44*$^{+/+}$ CD45.2^{+} and test *Usp44*$^{-/-}$ CD45.2^{+} cells to lymphocyte populations. Data are from four or five mice per group per experiment and were reproduced in two independent experiments, with the exception of secondary bone marrow transplantation, which was carried out in a single experiment. Bars represent mean ± SEM;

statistical analysis was done using the Student *t* test for two groups or ANOVA with Bonferroni post hoc test for multiple comparisons; no significant differences were found. NS=nonsignificant" (2).

3.3.3 *Usp44* is dispensable for B-cell-mediated immune response

"Given the high expression of *Usp44* in activated B cells (315), we further analyzed the responses of *Usp44*$^{-/-}$ B cells to *in vitro* stimulation and observed normal induction of activation markers and costimulatory molecules (Figures 3.3.5A and B), as well as normal cell proliferation, survival, and immunoglobulin class switching (Figures 3.3.5C–G). *Usp44*$^{-/-}$ mice also produced normal titers of antigen-specific antibodies of diverse isotypes following immunization (Figure 3.3.5H). Overall, we conclude that *Usp44*-deficiency does not impair B-cell function and antibody-mediated immunity" (2).

3.3.4 Effects of *Usp44* deficiency on B-cell lymphoma progression

"To study the effects of *Usp44*-loss on hematologic malignancy, *Usp44*$^{-/-}$ mice were crossed to the Eμ-Myc mouse line (286), which is highly susceptible to B-cell lymphoma due to cMyc overexpression in B cells. Although there was some trend toward earlier lethality of the *Usp44*$^{-/-}$ Eμ-Myc mice relative to the *Usp44*$^{+/+}$ Eμ-Myc group, the combined comparison among the *Usp44*$^{-/-}$, *Usp44*$^{+/-}$, and *Usp44*$^{+/+}$ Eμ-Myc genotypes did not reach statistical significance (Figure 3.3.5I)" (2). As such, we conclude that loss of *Usp44* did not significantly affect the onset and progression of B-cell lymphoma in Eμ-Myc mice.

Figure 3.3.5 Loss of USP44 does not impair B-cell activation, immunoglobulin class switching, or antibody production.

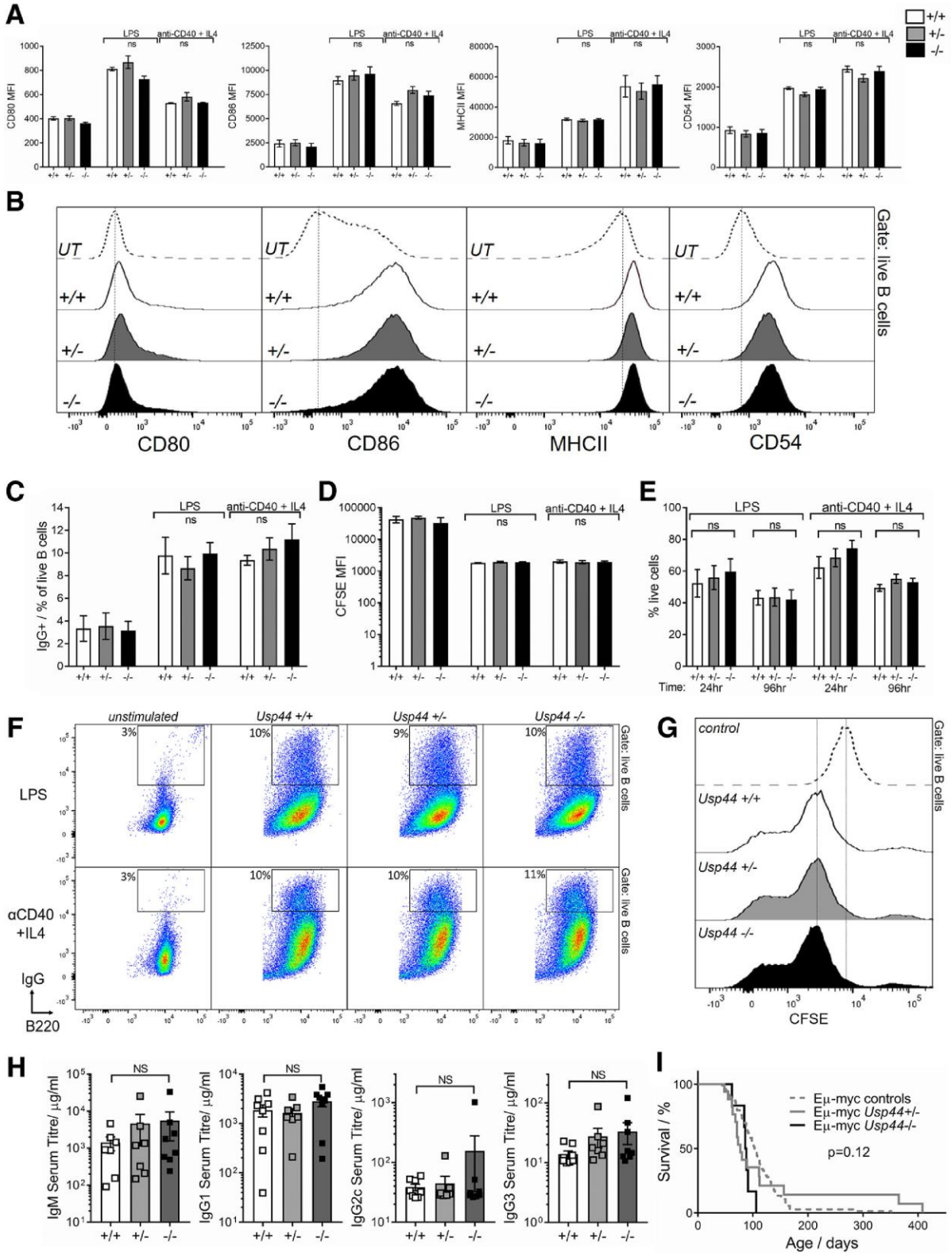


Figure 3.3.5 Loss of USP44 does not impair B-cell activation, immunoglobulin class switching, or antibody production. "**(A–G)** Analyses of the activation, class switching, proliferation, and survival of *Usp44*$^{+/+}$, *Usp44*$^{+/–}$, and *Usp44*$^{–/–}$ B cells isolated through magnetic enrichment from mouse spleen, stimulated *in vitro* with lipopolysaccharide (LPS; 1 μg/mL) or anti-CD40 (2 μg/mL) + interleukin-4 (5 ng/mL), and analyzed by flow cytometry, gating on live B cells (B220^{+}). **(A,B)** Bar charts and representative histograms showing expression of CD80, CD86, MHCII, and CD54 activation markers at 24 hours of stimulation. MFI=mean fluorescence intensity. **(B)** CD80 and CD86 data are from LPS stimulation, whereas MHCII and CD54 data are from CD40 + IL4 stimulation. **(C)** Percentage of IgG^{+} cells in the cultures at 96 hours of stimulation as a measure of immunoglobulin class switching. **(D)** Analysis of carboxyfluorescein diacetate succinimidyl ester (CFSE) staining intensity of the cells at 96 hours of stimulation as a measure of cell proliferation. **(E)** Percentage of live cells in cultures at 24 and 96 hours of stimulation. **(F)** Representative flow cytometry dot plots showing IgG^{+} class-switched B cells. **(G)** Representative histograms showing CFSE staining intensity of the cells at 96 hours of LPS stimulation as a measure of cell proliferation. **(H)** Titers of antigen-specific antibodies of the IgM, IgG1, IgG2c, and IgG3 isotypes in the serum of *Usp44*$^{+/+}$, *Usp44*$^{+/–}$, and *Usp44*$^{–/–}$ mice at day 14 after subcutaneous immunization with R-phycoerythrin in Complete Freund's Adjuvant. Data are from four to eight mice per group per experiment and were reproduced in two independent experiments, except for cell proliferation and immunization assays, which were carried out in a single experiment. Bars represent mean ± SEM; statistical analyses use ANOVA with Bonferroni post hoc test; no significant differences were found. NS=nonsignificant. **(I)** Survival of Eμ-Myc *Usp44*$^{+/+}$ (n = 80), Eμ-Myc *Usp44*$^{+/–}$ (n = 17), and Eμ-Myc *Usp44*$^{–/–}$ (n = 6) mice; p = 0.14, log–rank test; p = 0.12, Gehan–Breslow–Wilcoxon test (GraphPad Prism 7.01)" (2).

3.3.5 Potential functional redundancy of *Usp44* with *Usp49*

In previous studies using cell lines, loss of *Usp44* was linked to defects in gene transcription, DNA repair, and cell cycle transition (178, 198, 200, 272). All these cellular processes are known to be essential for HSCs, hematopoiesis, and lymphocyte development; yet surprisingly, we demonstrated that *Usp44* is dispensable in hematopoietic and immune systems *in vivo*. Therefore, functional redundancies between USP44 and other USP family members need to be explored.

"Phylogenetic analyses point to close homology of USP44 and USP49 (316-318) and the proteins also have many functional similarities; USP49 is also a nuclear protein (319) that can deubiquitinate histone H2B-K120ub (199), is recruited to DNA damage foci (320), and can interact with centrins (319). Further data mining (321, 322) and our qRT-PCR analyses indicate that *Usp49* is expressed in hematopoietic cells at similar or higher levels than *Usp44* (Figure 3.3.6 and Table 3.3.3) and normal *Usp49* expression persists in *Usp44*-knockout (Figure 3.3.6C). Future studies should explore possible functional redundancies between USP44 and USP49 to determine whether USP49 may compensate for USP44 loss in hematopoiesis" (2).

Figure 3.3.6 *Usp44* and *Usp49* gene expression.

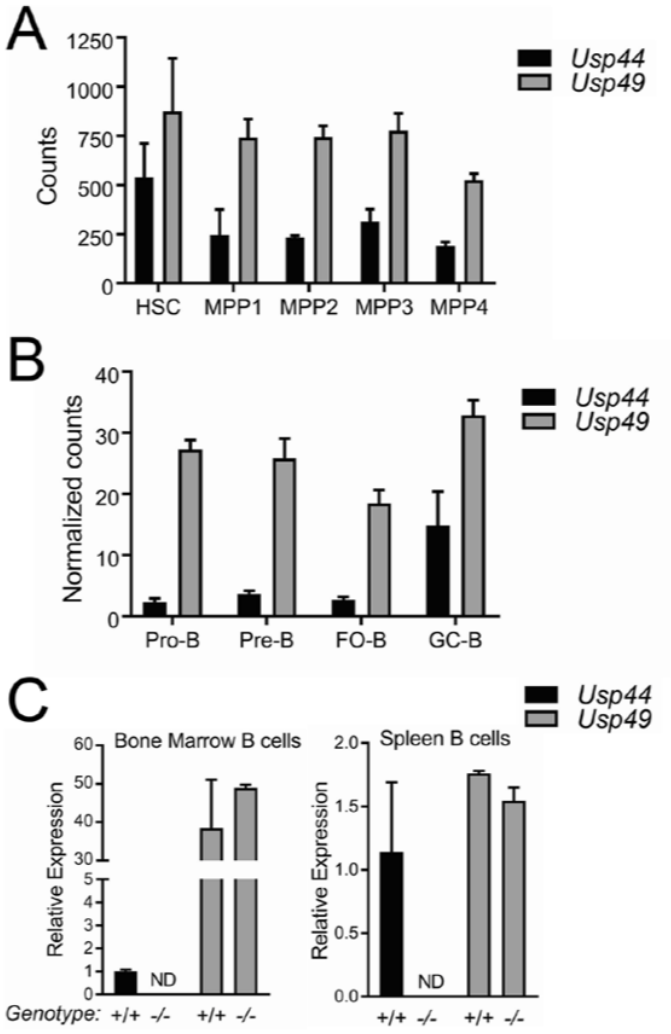


Figure 3.3.6 *Usp44* and *Usp49* gene expression. "**(A-B)** Expression of *Usp44* and *Usp49* genes in **(A)** hematopoietic stem cells (HSCs), hematopoietic multipotent progenitor cells (MPPs), and in **(B)** B cell lineage cells, based on previously published RNA-Seq data from these cell types in wild-type mice (321, 322). Expression was measured as **(A)** number of reads or **(B)** quantile normalized number of reads mapped to each gene; FO - follicular B cells, GC - germinal centre B cells. **(C)** Expression of *Usp44* and *Usp49* genes in B cells from the bone marrow and spleen of wild type and *Usp44*$^{-/-}$ mice, analyzed by qRT-PCR. Expression values for each tissue were normalized to β-actin and presented relative *Usp44*-expression in wild type samples. *Usp44* transcript levels in *Usp44*$^{-/-}$ samples were below the limit of detection; ND - not detected; similar results were obtained with three independent primer pairs per gene. All bars represent means +/- SEM from 2-4 independent biological samples" (2).

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 Summary

In this thesis, we characterized the roles of three deubiquitinating enzymes in different areas of normal and malignant hematopoiesis using mouse models. Our major novel findings are:

1) **BAP1 regulates cell cycle progression in pre-B cells of B lymphocyte development.**
    a) Using the cre-lox system, we generated an early B-cell-specific *mb1*-cre *Bap1*-knockout mouse model and analyzed the different stages of B-cell development within lymphoid organs, showing a cell-intrinsic requirement of BAP1 in maintaining splenic B cells.
    b) Within the bone marrow, BAP1-depletion resulted in a loss of large pre-B cells, associated with decreased cell viability and impaired cell cycle progression.
    c) Genome-wide transcriptome analysis revealed a downregulation of genes involved in cell cycle progression in BAP1-deficient pre-B cells; this defect was confirmed in pre-B cells and persisted in immature B and mature B cells.

2) **MYSM1-depletion protects against the tumorigenic activity of c-MYC in mice with c-MYC-driven B-cell lymphoma.**
    a) By crossing the Eμ-Myc mouse model of B-cell lymphoma with constitutive or tamoxifen-inducible *Mysm1*-knockout mice, we demonstrated a protective effect of *Mysm1*-loss in the survival of Eμ-Myc mice.

b) Transplantation of inducible *Mysm1*-knockout Eμ-Myc tumour cells into wild type recipient mice, followed by tamoxifen treatment to induce *Mysm1*-deletion in injected tumour cells, promoted disease remission and prolonged mouse survival.

c) Cultured *Mysm1*-deficient Eμ-Myc tumour cells exhibited decreased ribosomal protein gene expression, reduced protein synthesis rate, and increased p53 level.

3) USP44 is nonessential for the functions of hematopoietic stem cells, the development of lymphocytes, and the immune response mediated by B cells.

a) Using a knockout mouse model, we found normal frequency and function of hematopoietic stem and progenitor cells in *Usp44*-knockout mice.

b) With 6-7 Gy of whole-body irradiation, *Usp44*-knockout mice showed normal hematopoietic stress response, as assessed by monitoring mouse survival.

c) *Usp44*-knockout mice had normal lymphocyte development and produced normal levels of antigen-specific antibodies in response to immunization.

d) Isolated B cells from *Usp44*-knockout mice showed normal activation, cell proliferation, and immunoglobulin class-switch recombination response.

e) Loss of *Usp44* did not significantly affect the onset and progression of c-MYC-driven B-cell lymphoma in mice.

4.2 Regulation of B Lymphocyte Development by Deubiquitinase BAP1

In Chapter 3.1, "we established the non-redundant and cell intrinsic role of BAP1 in the normal progression of B cell development and suggest BAP1 as a regulator of the transcriptional programs required for cell proliferation and cell cycle progression in pre-B cells" (1).

"B cell dysfunction was previously reported by Arenzana et.al., following an inducible deletion of *Bap1* in the *Bap1*$^{fl/fl}$*Cre*^{ERT2} mouse model (255), showing a depletion of pre-pro-B, small pre-B, immature, and mature B cell subsets. It is, however, important to note the complementarity and the differences between our experimental systems, with Arenzana et.al. analyzing an acute and systemic loss of BAP1 (234, 255), whereas our current work assesses BAP1 deletion specifically within the B cell lineage (285). Such differences likely account for the differential depletion of the B cell precursor subsets across the two models. Overall, our work for the first time allows to assess the cell intrinsic role of BAP1 following B cell lineage commitment, independently of its role in the earlier hematopoietic progenitor cells or the cells of the bone marrow niche (234, 255)" (1).

"While our study links the depletion of large pre-B cells in *Bap1*$^{fl/fl}$ mb1-*Cre* mice to the disruption in cell proliferation and the direct role of BAP1 in the regulation of genes required for cell cycle progression, the expansion of Fraction B pro-B cells also seen in *Bap1*$^{fl/fl}$ mb1-*Cre* mice is not linked to changes in cell cycle or cell survival, and remains to be further explored in future work. Similarly, the mechanisms leading to the depletion of mature B cells in the bone marrow, spleen, and lymph nodes of *Bap1*$^{fl/fl}$ mb1-*Cre* mice remain to be further addressed and may be distinct from the mechanisms driving the depletion of large pre-B cells, analyzed in our current study. In particular, the cross-talk between BAP1 functions in mature B cells and the

BAFF/BAFFR pathway essential for the survival of transitional-2, follicular and of marginal zone B cells (323, 324), and the NOTCH pathway required for marginal zone B cell development (325), merit further investigation. Interestingly, the role of BAP1 as a regulator of NOTCH pathway signaling is already indicated in other cell types (326). Furthermore, the role of BAP1 in the development and maintenance of B1 cells is not sufficiently addressed in our current work, and requires further investigation, given that B1 cell development is driven by distinct progenitor cells, occurs in distinct developmental waves during ontogeny, and is regulated by distinct transcription factors and gene expression networks (327, 328)" (1).

"Our work has also advanced the understanding of the molecular functions of BAP1 within the B cell lineage. Our findings suggest that BAP1 may have a direct role in the regulation of the transcriptional programs of cell cycle progression in B cell development. Although our transcriptional analyses of *Bap1*$^{fl/fl}$*Cre* pre-B cells is somewhat confounded by the depletion of the more proliferative large pre-B cell subset from the analyzed cell population, it is important to note that the dysregulation in cell cycle was observed in both large pre-B and small pre-B cell subsets that make up the pre-B cell pool sorted for the transcriptional profiling" (1). Other studies conducted by my colleagues on this project demonstrated direct binding of BAP1 to the promoters of genes needed for normal cell proliferation and cell cycle progression (Liang Y, Wang H, data not shown) (1). Furthermore, loss of BAP1 resulted in increased H2A-K119ub levels at these genes (Liang Y, Wang H, data not shown) (1). These findings further support the conclusion that BAP1 directly regulates the expression of genes required for normal cell cycle progression in pre-B cells (1). "Overall, we hypothesize that a disruption in BAP1-regulated transcriptional programs of cell proliferation and cell cycle progression may contribute to the defects in B cell development in BAP1-deficiency" (1).

"The functional link between the loss of BAP1 and impaired cell proliferation, suggested for the B cell lineage in our current work, is shared across several different cell types (255, 301-304). As already discussed, an inducible systemic deletion of *Bap1* in mice results in impaired proliferation of thymocytes and peripheral CD4 T cells (255), suggesting shared BAP1 function across the different cell types of the lymphoid lineage. Depletion of erythrocytes and platelets was also reported in this model; however, it is not linked to specific cell proliferation defects (234). BAP1 was also shown to be essential for proliferation of several non-hematopoietic cell lines, regulating transcriptional programs of cell cycle progression in complex with HCF1 (241, 243, 301-304). Importantly, however, the role of BAP1 as a positive regulator of cell maintenance and proliferation is not universal, and *Bap1* deletion in mice results in an expansion of myeloid leukocytes and progenitor cells, resembling the pathology of MDS and CMML (234)" (1). "The mechanisms resulting in the distinct outcomes of BAP1 loss on the myeloid and lymphoid cell lineages merit further investigation, and may provide insights into the functions of BAP1 and its ASXL binding partners as tumour suppressors (240, 329, 330)" (1).

"BAP1 is an important tumour suppressor, and germline *BAP1* mutations in human result in a strong predispositions to a range of cancers, including mesothelioma, uveal melanoma, renal cell carcinoma, and others (240, 329). Although B cell lymphomas are not commonly associated with *BAP1* mutations, cases of non-Hodgkin lymphoma have been reported in carriers of germline *BAP1* mutations (331). Furthermore, lymphomas that carry other genetic aberrations have been shown to acquire *BAP1* gene silencing via epigenetic mechanisms (332). This raises the possibility that BAP1 may function as a tumour suppressor also in the B cell lineage. This hypothesis remains to be further tested in mouse models, for example by screening for the incidence of spontaneous lymphomas in $Bap1^{fl/fl}$ *Cre* mice, or by tracking other age-associated phenotypes and the life-span

of the animals. Future studies will need to further address these questions, providing insights into potential tumour suppressor functions and other functions of BAP1 within the B cell lineage" (1).

"The molecular mechanisms underlying the tumour suppressor activities of BAP1 remain controversial, especially in the light of this and many other reports implicating BAP1 as a positive regulator of cell proliferation (303). The tumour suppressor activity of BAP1 in myeloid progenitor cells was linked to its antagonism with PRC2, as the transformation of BAP1-deficient myeloid cells was associated with an increase in PRC2-mediated transcriptional repression and could be abolished by the loss or inhibition of the PRC2 catalytic subunit EZH2 (333, 334). Similar mechanisms are implicated in the tumour suppressor activities of BAP1 in mesothelioma (333), but not in uveal melanoma cell lines (335). Other mechanisms linking BAP1 molecular functions and its tumour suppressor activity are also proposed, including: its roles in transcriptional regulation of pro-survival and pro-apoptosis transcriptional programs in cross-talk with PRC1 (336); its role as a positive regulator of ferroptosis via repression of *SLC7A11* gene expression (203); its role in the transcriptional regulation of cellular metabolic programs (249, 337-339), and in the regulation of Ca^{2+} signaling and apoptosis via deubiquitination and stabilization of receptor-channel IP3R3 (340, 341). We observed that *SLC7A11* was not expressed in pre-B and immature B cells, from either control or $Bap1^{fl/fl}Cre$ mice, indicating that the regulation of ferroptosis by BAP1 is unlikely to play significant physiological role in these B cell precursor cells" (1). "Future studies will need to further address the molecular mechanisms underlying the tumour suppressor activities of BAP1 in different cell types, in the light of its apparent positive role in the regulation of cell proliferations in non-transformed cells of many tissues" (1).

"We have demonstrated that the loss of BAP1 results in impaired B lymphocyte development, and suggested the link of this phenotype to the altered transcriptional profile and the

defects in proliferation in BAP1-deficient pre-B cells. It is important to note, however, that BAP1 remains expressed throughout the B cell lineage, and that cell cycle analysis in *Bap1*$^{fl/fl}$ *Cre* mice revealed significant changes not only in pre-B cells, but also in immature and mature B cells. It is therefore highly likely that BAP1 remains engaged as an important transcriptional regulator throughout B cell ontogeny. Cell proliferation is essential not only for the formation and maintenance of the pool of naïve B cells, but also for the normal execution of B cell mediated immune response, including clonal expansion during the germinal centre reaction (36, 342, 343). Future studies should address the cell intrinsic role of BAP1 in B cells during the execution of humoral immune response, including the germinal centre reaction, and the differentiation and persistence of plasma and memory B cells" (1).

"In summary, our work establishes the non-redundant and cell intrinsic role of BAP1 in B cell development. It further suggests a direct role of BAP1 in the induction of the transcriptional programs of cell proliferation and cell cycle progression in pre-B cells, linked to its catalytic function as a deubiquitinase for histone H2AK119ub" (1).

4.3 Therapeutic Applications of Targeting Deubiquitinase MYSM1

In Chapter 3.2, we demonstrated that MYSM1 is needed for c-MYC to fulfill its oncogenic activities in B-cell lymphoma. Depletion of the entire MYSM1 protein disrupted the overexpression of ribosomal protein genes that was driven by c-MYC, increased the protein levels of p53, and led to a protective effect in mouse models of B-cell lymphoma. These findings advance our understanding of MYSM1 in c-MYC-driven B-cell lymphoma and suggest MYSM1 as a potential therapeutic target for c-MYC-driven hematologic malignancies.

Future studies may examine whether losing only the deubiquitinase activity of MYSM1 reproduces these beneficial effects in hematologic malignancies, prolonging survival and inducing disease remission. As advancement towards the clinic depends on our research findings being translated to humans, future studies may also examine the effects of MYSM1-loss in human c-MYC-driven lymphoma cells. Since MYSM1 performs similar essential functions in normal hematopoiesis of both mice and humans, the effects of MYSM1-depletion on B-cell malignancies may be conserved between mice and humans (229, 230, 257-259, 269). Altogether, this knowledge will provide a rationale for the development of a chemical inhibitor against the catalytic activity of MYSM1 as a potential therapeutic treatment for c-MYC-driven B-cell lymphoma. Drugs targeting other members of the zinc metalloproteinase family have entered clinical trials (344), suggesting that the development of MYSM1 small-molecule antagonists for *in vivo* use may be feasible. Our data lends credence to the hypothesis that such compounds may repress cMYC-driven expression of ribosomal and translational machinery, and may therefore synergistically enhance the efficacy of inhibitors that directly target the ribosome, in development for cancer chemotherapy (345, 346).

Our findings in Chapter 3.2 suggest another approach for indirectly targeting the effects of c-MYC in c-MYC-overexpressing blood cancers, including most Burkitt lymphoma cases and more aggressive diffuse large B-cell lymphoma cases (42, 43). These hematologic malignancies aberrantly express c-MYC through *c-Myc* gene rearrangement or amplification and rely on a continuous source of c-MYC for survival; however, a direct attack on the c-MYC protein proves to be complicated as this transcription factor localizes to the nucleus and lacks a ligand-binding active site (40-46). Current strategies involve blocking *c-Myc* gene transcription or protein translation, as well as disrupting protein-protein heterodimerization or DNA-binding of c-MYC and its partner MAX (40, 41, 44). Other useful methods target the downstream effects of c-MYC-overexpression, such as hyperactive ribosome biogenesis via suppression of RNA polymerase I that synthesizes ribosomal RNA (347). A small molecule inhibitor of RNA polymerase I, CX-5461, was shown to induce nucleolar stress and p53-dependent apoptosis or cell cycle arrest along with caspase-dependent apoptosis in various hematologic cancer cells of mouse and human origins, while leaving normal murine B cells alive (347-350). Recently, in phase I of the first clinical trial with 16 advanced stage blood cancer patients, CX-5461 inhibited ribosomal RNA gene transcription in most cases and demonstrated stable or beneficial responses in some cases (351). In the patient showing partial response, p53 activation was also observed in tumour tissue after CX-5461 treatment (351). As these researchers recruited patients at advanced stages of disease who have undergone standard therapies to no avail, it is likely that patients at earlier stages may respond more robustly (351, 352). Together, preclinical studies and the phase I trial reveal promising results for targeting ribosome biogenesis in human hematologic malignancies (351). Future studies may examine whether blocking MYSM1 in combination with inhibiting RNA polymerase I demonstrate therapeutic efficacy in blood cancers.

In addition to affecting hematologic cancers, CX-5461 suppressed the growth of non-hematologic diseases with high expression of c-MYC, for instance small cell lung cancer, prostate cancer, and neuroblastoma, in preclinical studies and is currently in phase I of a clinical trial for patients with solid tumours (Canadian Cancer Trials Group, NCT02719977) (352-355). These results indicate the effectiveness of attacking ribosome biogenesis for other varieties of c-MYC-driven cancers. Discovery of potent c-MYC-targeting strategies may, therefore, improve the treatment of multiple human cancers with c-MYC-dysregulation.

4.4 Functional Redundancy of Deubiquitinase USP44 in HSC and B Lymphocyte Functions

In Chapter 3.3, we reported that "USP44 is dispensable for HSC function under homeostatic and stress conditions, for lymphocyte development, and also for B-cell activation, immunoglobulin class switching, and antibody-mediated immune response. *Usp44* loss also did not have significant effects on Eμ-Myc B-cell lymphoma onset and progression. This is consistent with previous reports showing that aged *Usp44*$^{-/-}$ mice have an elevated incidence of lung adenoma, but only a mild increase in lymphomas (273)" (2). "USP44 was previously implicated as an important regulator of cell cycle progression, gene expression, and DNA repair using studies *in vitro* and in cell lines (178, 272, 356). Nevertheless, normal viability of *Usp44*$^{-/-}$ mice reported here and previously (273) suggests that USP44 functions may be redundant with other USP family proteins or may be cell-type restricted" (2).

Mice deficient for histone H2A deubiquitinase USP21 also showed normal hematopoietic stem cell function and lymphocyte development; the actual roles of these USP proteins may have been masked by other functionally similar histone deubiquitinases (2, 239). Important histone deubiquitinases involved in hematopoiesis include MYSM1, BAP1, USP3, USP16, and USP22, which participate in regulating gene expression, DNA damage response, and cell cycle, contributing to hematopoietic stem cell self-renewal, downstream cell differentiation, and cell development (27, 189, 220, 225, 229-234, 241, 255, 261-263, 305, 306, 333, 357). Functional redundancy within the hematopoietic stem cell and lymphocyte compartments would suggest the importance of maintaining ubiquitinated histone levels at these stages of blood cell development.

From our literature search emerged a potential protein candidate: USP49, which is a close homolog of USP44 and shares many functions (2, 316-318). USP49 also localizes to the nucleus, interacts with centrin proteins, associates with sites of DNA damage, and removes histone H2B-K120ub (199, 319, 320). Analysis of published RNA-seq data combined with our RT-qPCR results revealed a similar or higher expression of *Usp49*, compared to that of *Usp44*, in hematopoietic cells from wild type mice; in addition, *Usp44*-knockout splenic B cells continued to express normal levels of *Usp49* (2, 321, 322). Future experiments may determine whether USP44, USP49, and/or other family members are functionally redundant or cooperate in the regulation of hematopoietic stem cell and lymphocyte development, for example, by comparing hematopoietic cell functions of *Usp44*- and *Usp49*-double knockout mice with single knockout mice to reveal whether USP49 compensated for the loss of USP44 (2).

Following the publication of our journal article, another group, Zhang et al. (2020) studied the function of USP44 in the antiviral innate immune response, discovering the importance of USP44 in the protection against DNA viruses (358). *Usp44*-knockout mice were more prone to death that is induced by intraperitoneal infection of herpes simplex virus 1 (HSV-1; a DNA virus), but not vesicular stomatitis virus (VSV; an RNA virus), relative to control mice (358). This increased susceptibility of *Usp44*-knockout mice to HSV-1-induced death was associated with decreased type I interferon and proinflammatory cytokine levels, elevated viral titers, and more tissue damage compared to control mice following HSV-1 infection (358). Further experiments at the molecular level showed that USP44 deubiquitinates K48-linked polyubiquitin chains on mediator of IRF3 activation (MITA; also called stimulator of interferon genes, STING), preventing the proteasome-mediated degradation of MITA/STING and stimulating activation of the innate immune response against DNA viruses (358). In this study, the authors identified MITA/STING

as a substrate of USP44 by examining the extranuclear functions of USP44 in the cytoplasm and membranes (358). Together, our work and their paper uncovered the role of USP44 in hematopoietic stem cell functions, lymphocyte development, and innate immune response to viral infections (2, 358). “USP44 functions in other physiologic systems beyond hematopoiesis merit further investigation. Indeed, the *Usp44*$^{-/-}$ mouse strain is undergoing extensive phenotypic characterization of other physiologic systems with IMPC and the resulting data may serve as a foundation for further studies (www.mousephenotype.org/data/genes/MGI:3045318#section-associations)” (2).

4.5 Conclusion: Expanding Our Knowledge of Transcriptional Regulation in B Cells

In Chapter 3.1, we explored the role of deubiquitinase BAP1 in B cells with a B-cell-specific *Bap1*-knockout mouse model. To avoid potential confounding effects arising from BAP1-loss in earlier hematopoietic and lymphoid progenitor cells or in supportive cells within the B-cell niche, we specifically deleted *Bap1* upon B-cell lineage commitment. Our work examined the previously uncharacterized molecular functions of BAP1 in B cells. Consistent with its activities reported in other hematopoietic lineages as well as in other cell types, BAP1 is also involved in the regulation of cell cycle in B cells (255, 301, 303, 304). We demonstrated that loss of BAP1 in B cells led to a downregulation of cell proliferation and cell cycle genes in pre-B cells, along with impaired cell cycle progression starting from the pre-B cell stage; this molecular phenotype was associated with hindered cell development within the bone marrow that persisted in secondary lymphoid organs. Our findings suggest a role for BAP1 in the transcriptional regulation of B cells, particularly in the control of cell proliferation and cell cycle progression required for B-cell development.

In Chapter 3.2, we investigated the role of deubiquitinase MYSM1 in c-MYC-driven B-cell lymphoma using mouse models. Previous studies have elucidated the importance of MYSM1 in transcriptional activation of developmental genes in various blood cells during normal hematopoiesis (including *Ebf1* in B-cell progenitors) (229, 261-263, 305, 306). Recently, our lab has also characterized the role of MYSM1 in regulating the expression of ribosomal protein genes and translation-associated genes in hematopoietic stem and progenitor cells (269). Similarly, here, we found that loss of MYSM1 affected the transcription of c-MYC-dependent ribosomal protein genes in Eμ-Myc B-cell tumour cells. Dysregulation of gene expression in *Mysm1*-depleted Eμ-Myc cells was associated with decreased level of elongation factor 1-gamma (EEF1G) protein,

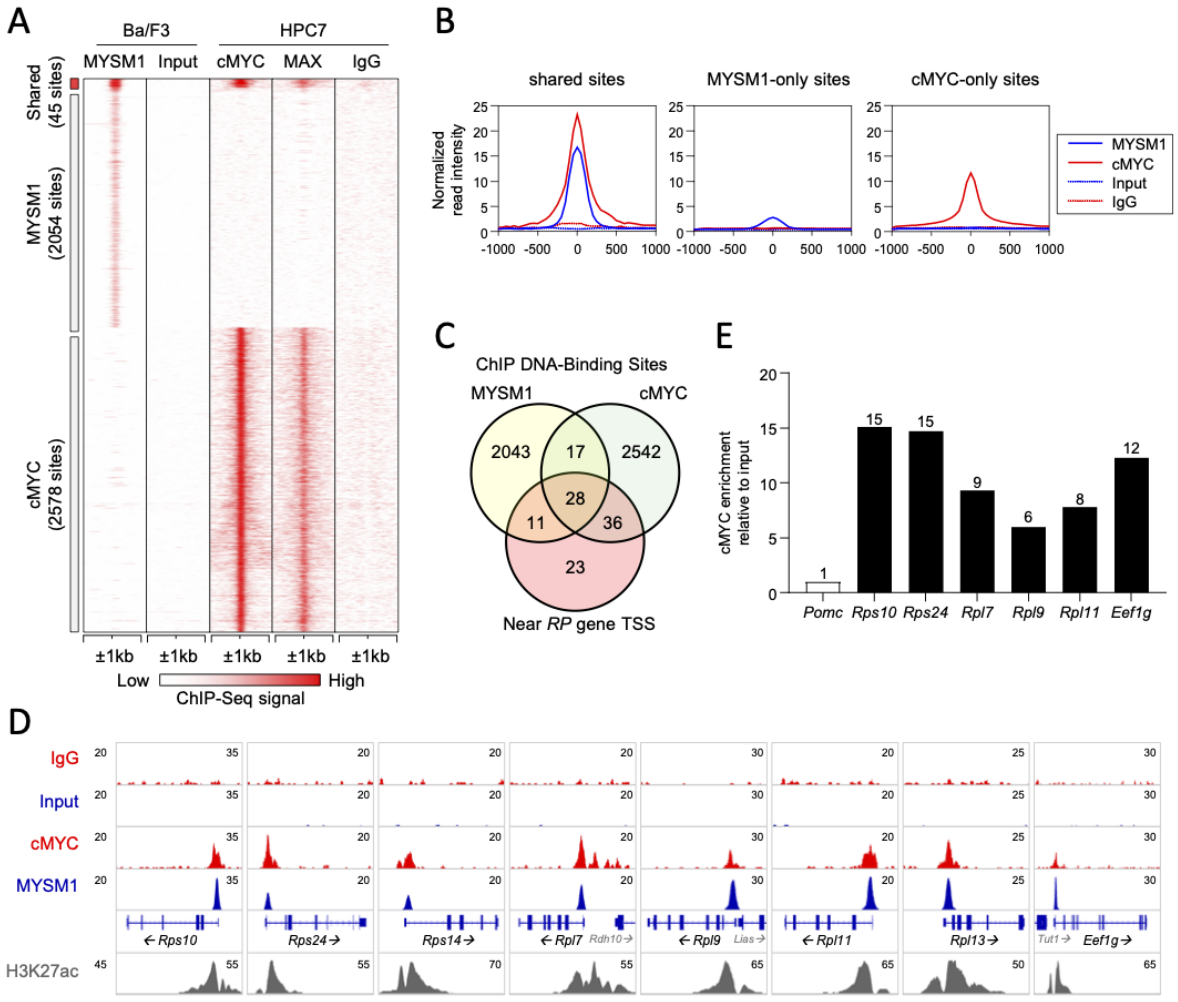
reduced rate of protein synthesis, and upregulated level of p53 protein. Using mouse models, we showed that loss of MYSM1 in Eμ-Myc tumour cells upon transplantation into recipient mice promoted disease remission. Altogether, our study was the first to examine MYSM1 in the context of hematologic malignancies and supported the role of MYSM1 in transcriptional regulation of ribosomal protein genes in c-MYC-driven B-cell lymphoma.

In Chapter 3.3, we focussed on deubiquitinase USP44 in the blood and immune systems of mice as USP44 was shown to participate in key cellular functions such as cell cycle progression, DNA repair, and gene transcription (178, 198, 200, 272, 273). We reported that USP44 is nonessential in normal development of B cells and B-cell-mediated immune response, which require these functions for activation, proliferation, differentiation, and class-switch recombination (2). The functions of USP44 in transcriptional regulation of B cells are perhaps shared by a group of histone deubiquitinases containing BAP1, MYSM1, and/ or other USP enzymes, some of which having more noticeable contributions.

In conclusion, this thesis examines the roles of three histone deubiquitinases in the hematopoietic system, particularly the B-cell compartment, and revealed a shared function of transcriptional regulation in B cells. BAP1 regulates the transcriptional program of cell cycle progression and proliferation in pre-B cells, whereas MYSM1 is involved in the regulation of ribosomal protein genes in malignant c-MYC-driven B-cell lymphoma cells. On the other hand, USP44 may be functionally redundant in B cells. Future studies may characterize other histone deubiquitinases in B cells and elucidate their potential in regulating the blood system.

CHAPTER 5: APPENDIX

Appendix Figure 5.1 for Chapter 3.2: Co-localization of MYSM1 and cMYC DNA binding sites at the promoters of genes encoding ribosomal proteins (*RPs*).



Appendix Figure 5.1 for Chapter 3.2: Co-localization of MYSM1 and cMYC DNA binding sites at the promoters of genes encoding ribosomal proteins (*RPs*).

(A) Consolidation of genome-wide DNA-binding data for MYSM1, cMYC, and MAX using ChIP-seq datasets from Belle et al., *JCI Insight* 2020 (MYSM1) (269) and Wilson et.al., *Blood* 2016 (cMYC, MAX) (308), highlighting 45 shared binding sites between MYSM1 and cMYC/MAX. Input and IgG served as background controls for the two cell lines. **(B)** Histograms showing the average normalized read intensities of MYSM1 and cMYC around the shared, MYSM1-only, and cMYC-only binding sites, from the ChIP-seq datasets above. **(C)** A Venn diagram comparing MYSM1 and cMYC DNA-binding sites, and showing the number of binding sites within 1kb of a transcription start site (TSS) of a ribosomal protein gene (*RP* gene). This demonstrates that at least 28 *RP* gene promoters carry overlapping MYSM1 and cMYC binding sites; please note that we don't exclude that MYSM1 and cMYC may also regulate other *RP* genes, not picked up in these ChIP-seq datasets for technical reasons. **(D)** Genomic snapshots of cMYC and MYSM1 binding near select *RP* gene promoters. Data for MYSM1 is from Belle et al., *JCI Insight* 2020 (269); data for cMYC is from Wilson et.al., *Blood* 2016 (308). **(E)** Validation of cMYC binding at known MYSM1 DNA-binding sites at the promoters of genes encoding ribosomal proteins and translation factors, in a B cell precursor cell line Ba/F3 using ChIP-qPCR; data presented is from a representative experiment and was reproduced in five or more independent experiments for each genomic region. All Ct values were normalized to those of the pro-opiomelanocortin (*Pomc*) gene, which serves as a negative binding region. Enrichment was calculated relative to input DNA.

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