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Function of Deubiquitinase MYSM1 in Attenuation of DNA Damage Responses

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by
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Brendan Mathias

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August 2023

Dedicated to my parents.

ABSTRACT OF THE DISSERTATION

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MYSM1 deficiency has been identified in patients with B cell lymphopenia, hypogammaglobulinemia, primary immune deficiency and bone marrow failure. MYSM1 is a known deubiquitinase that acts on histone H2A to promote gene expression. MYSM1 also functions in cellular responses to DNA injury and patients with loss of MYSM1 have increased sensitivity to DNA damaging agents. The specific role and function of MYSM1 in DNA damage responses has yet to be determined. Here, I have shown that MYSM1 does not mediate generation or repair of DSBs, but rather acts to attenuate and terminate DNA damage signaling after repair. Loss of MYSM1 results in persistent DNA damage response signaling characterized by persistent 53BP1 foci and ATM activity, whereas MYSM1 overexpression results in premature DDR resolution without break repair. These results demonstrate that MYSM1 plays an important role in the resolution of DDR signaling, a vital component of genomic integrity and lymphocytic development.

Chapter 1

Introduction

Our lab first became interested in studying MYSM1 when a newborn baby presented with B cell lymphopenia resulting from a mutation in MYSM1. B cells are an essential component of a healthy immune system, and the role of MYSM1 in B cell development or maintenance had not yet been explored in depth.

1.1 B Cell Development

As a component of adaptive immunity, B cells are responsible for producing antibodies necessary to fight infection and other deleterious agents. B cells are hematopoietic cells that begin development in the bone marrow. An important stage of B cell development includes deliberate generation of programmed DNA double-strand breaks (DSBs) to assembly the genes needed for generation of a diverse repertoire of antibodies.

1.1.1 The role of B cells in the immune system

B cells fulfill an important role in the immune system by producing antibodies and maintaining antigen memory. Antibodies are first expressed as B cell surface receptors, also known as antigen receptors. Antigens are small molecular components of pathogens that can activate an immune response. Antibodies will bind to any pathogens that present their target antigen and either neutralize pathogenic activity (neutralizing antibodies) or designate the pathogen for degradation by other immune system components.

A mature B cell expresses a unique antigen receptor that is specific to a single antigen. When an immature B cell in the lymph node encounters an antigen that activates its receptor, it is stimulated to both replicate and produce more antibodies that will bind to the target antigen (Murphy & Weaver, 2017). Upon stimulation by an activating antigen, a B cell will activate a variety of cellular processes including proliferation and antibody secretion. These secreted

antibodies bind their designed target to neutralize it or designate it for degradation by other components of the immune system.

After the infection has been cleared, some remaining B cells develop into memory B cells to maintain antibody recall. The maintenance of these memory B cells and their associated antibodies allows the immune system to protect against reinfection and mount a faster and more effective response on future encounters with target antigen/pathogen. This is the foundational principle of vaccination. By presenting the immune system with attenuated pathogenic antigens, we can train the immune system and B cells, in particular, to recognize these antigens and develop protective antibodies. Upon infection by the more virulent strain of a pathogen, the immune system is already trained and prepared to defeat it with significantly more success than a primary infection.

1.1.2 Early B cell development

B cells begin development through differentiation from hematopoietic stem cells (HSCs) in the bone marrow. HSCs proceed through development in a stepwise fashion to produce a variety of different cell types. The common lymphoid progenitor (CLP) stage is responsible for the development of the adaptive immune system. At this stage, different transcription factors are activated that direct the cell into either B or T lymphocyte lineages. Activation of the transcription factors PAX5, EBF1, PU.1, Ikaros, and E2A promote differentiation to B cells (Vilagos et al., 2012). These five transcription factors cooperate to commit progenitor cells to the B cell lineage promoting differentiation of CLP to the prepro-B cell stage (Mandel & Grosschedl, 2010). B cell development then proceeds in a stepwise fashion through a series of defined stages until reaching the immature B cell stage (Fig 1.1).

Following B cell specification, the next stages of development coordinate assembly of the B cell antigen receptor, which is required for continued B cell development and for B cell function. The number of pathogenic agents far outweighs the number of genes in the human genome, so antibody genes cannot be encoded directly. To overcome this limitation, antigen receptor diversity is induced through recombination of antigen receptor (i.e., antibody) gene to vastly expand the antibody repertoire.

Recombination and assembly of the antigen or immunoglobulin receptor (*Ig*) requires generation and repair of programmed DSBs to facilitate expression of the mature antigen receptor. The antigen receptor is comprised of two proteins: the heavy chain (*IgH*) immunoglobulin and the ligh chain (*IgL*) immunoglobulin, which are assembled and expressed at distinct developmental stages. *IgH* gene is rearranged at the pro-B stage, and *IgL* gene is rearranged at the pre-B stage (Fig 1.1). Once the cell leaves the prepro-B stage and enters the pro-B stage of development, recombination of IgH is initiated (Helmink & Sleckman, 2012; Kim et al., 2015; Teng & Schatz, 2015). Successfully expressed IgH associates with a surrogate light chain (VpreB). Together, these two proteins form the pre-B cell receptor (pre-BCR). Pre-BCR signaling triggers proliferation and clonal expansion of large pre-B cells. After this proliferative burst, cells exit the cell cycle and transition to the small pre-B cell stage, where *IgL* recombination is initiated. Pairing of IgH and IgL forms the B cell receptor (BCR). Signaling through the BCR triggers emigration out of the bone marrow and into peripheral lymphoid organs, specifically to lymph nodes and spleen.

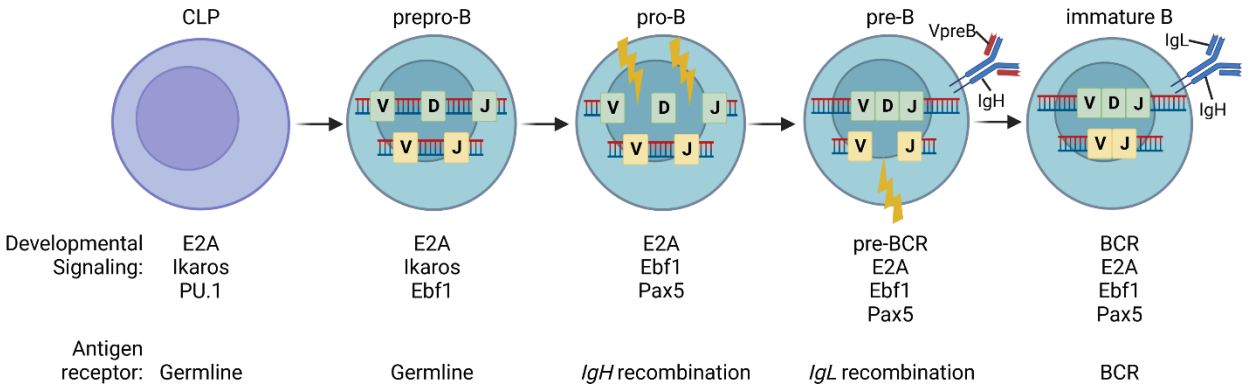

Figure 1.1 B cell development. Cells are committed to the B cell lineage with expression of E2A and EBF1 transcription factors. At the pro-B stage, V(D)J recombination occurs at *IgH* to create the mature IgH. Expression of *IgH* with *VpreB* forms the pre-BCR. Pre-BCR signaling promotes transition to the pre-B stage. Pre-B cells initially undergo a proliferative burst, then trigger recombination at *IgL* to form the mature IgL. Expression of both IgH and IgL chains forms the mature B cell receptor and promotes transition to immature B cell stage. Immature B cells then migrate out of the bone marrow for further development. Created with Biorender.com.

1.1.3 V(D)J recombination

The diversity of B cell antigen receptors is encoded through gene recombination. Germline antigen receptor genes contain a non-functional second exon that contains two-to-three regions with multiple potential gene segments in each of these regions. These segments are known as the variable (V), diversity (D), and joining (J) regions of the antibody gene locus. Recombination of these gene segments, paired with an error-prone repair process, allows for sufficiently large antibody diversity. The *IgH* gene contains V, D, and J segments, whereas the *IgL* genes contain only V and J gene segments. The process of recombination of these antibody gene regions is known as V(D)J recombination and is exclusive to developing B cells. V(D)J recombination is essential for B development, maturation, and diversification. Errors in the process block B cell differentiation with resultant B cell lymphopenia, hypogammaglobulinemia (low antibodies), and immune deficiency.

V(D)J recombination is initiated by the recombination activating gene endonuclease complex (RAG) comprised of a heterodimer of two RAG1 and two RAG2 proteins (Fig. 1.2). This complex is exclusively expressed in developing B and T cells. RAG binds DNA at two recognition signal sequences (RSS) in antigen receptor genes and cleaves the DNA to generate two double-stranded DNA breaks (DSBs). The DNA ends of these two breaks are then localized to each other. Repair is mediated through non-homologous end joining (NHEJ), an error-prone mechanism that directly ligates broken DNA ends together. NHEJ repair generates both a coding join contained in the mature antibody gene, and a signal join contained in the excised DNA.

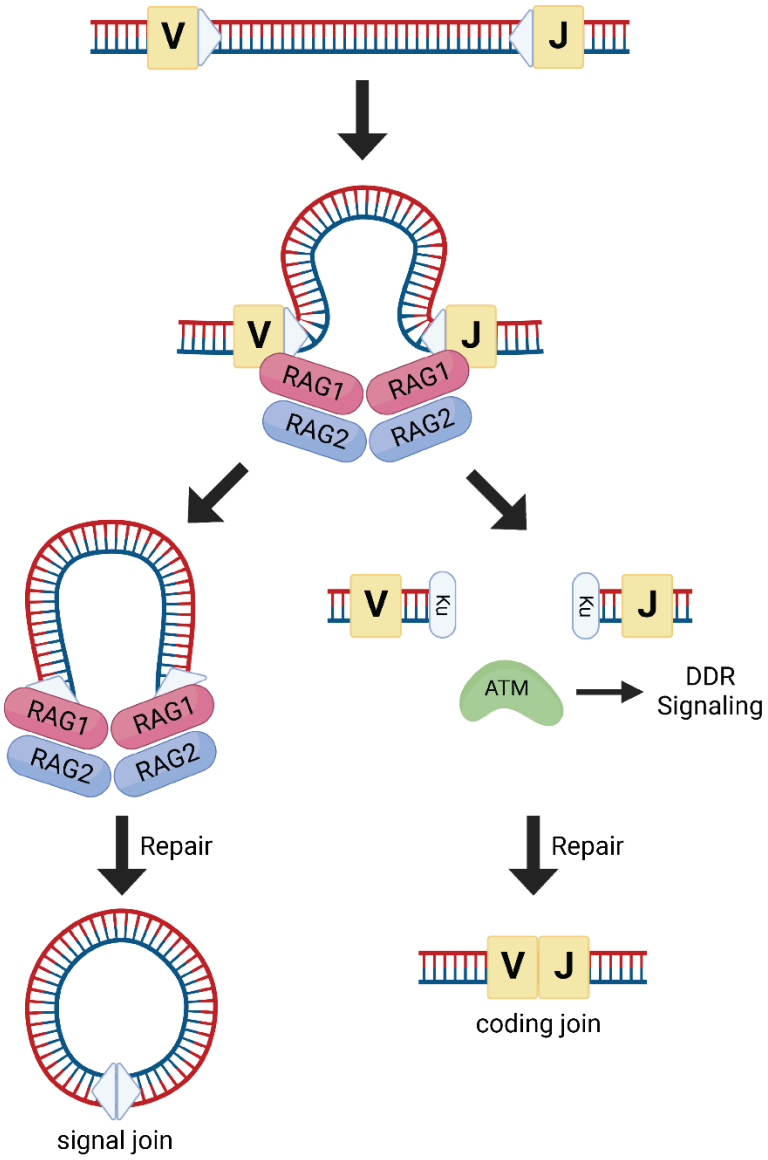

Figure 1.2 V(D)J recombination. The RAG endonuclease complex, comprised of a heterodimer of two RAG1 and two RAG2 proteins, binds to DNA at two recognition signal sequences (RSS) and generates two DSBs. The broken DSBs are joined by NHEJ. ATM activates NHEJ to repair the breaks while RAG sequesters the unnecessary signal ends. Created with Biorender.com.

1.2 DNA Damage Responses

DNA damage is highly deleterious to cell survival, so effective monitoring of damage and appropriate repair is paramount. DNA can suffer damage in a variety of ways, and cells have a robust response to ensure proper recognition of damage and error-free repair.

1.2.1 Sources of DNA damage

DNA damage can be generated by both exogenous and endogenous agents. Exogenous DNA damage comes from external agents including UV radiation or chemotherapy and generally is toxic to the genome. This type of damage occurs randomly at any stage in the cell cycle. Endogenous DNA damage comes from within the cell and includes errors of replication, oxidizing agents generated during metabolism (reactive oxygen or nitrogen species), or deliberate programmed breaks. These types of endogenous damage can be cell cycle-specific. Replication injury can only occur in proliferating cells, whereas metabolic damage can occur in proliferating or arrested cells responding to infection.

Regardless of mechanism of injury, DSBs trigger a conserved cellular response to ensure proper repair or activation of cell death if not repaired. However, in the case of programmed RAG-mediated DSBs, a second cell type-specific developmental response is also initiated (Bednarski et al., 2016; Johnston et al., 2023).

1.2.2 Mechanisms of DNA repair

There are several methods for repairing DSBs. The most common processes are homologous recombination (HR) and non-homologous end-joining (NHEJ). HR is an error-free process that utilizes the sister chromatid as a template for the repair of the DSB, ensuring error-

free repair. Consequently, this method of repair is only available during or after DNA replication. When DSBs occur in G1 before replication when no sister chromatid is available, or in instances of rapid repair, the error-prone process of NHEJ is employed (Shibata & Jeggo, 2020a). NHEJ is also the sole mechanism used for the repair of programmed breaks during antigen receptor gene assembly, and will be the focus of this next section.

1.2.3 Non-homologous end-joining and DNA damage signaling

In developing lymphocytes, NHEJ facilitates repair of RAG-mediated DSBs. After a DSB occurs, DNA ends are bound by Ku to prevent further damage and keep broken DNA ends in close proximity (Shibata et al., 2018). The MRN complex, consisting of MRE11, RAD50, and NBS1, senses the DSBs and recruits the serine-threonine kinase ataxia telangectasia-mutated (ATM). As a serine/threonine kinase, ATM phosphorylates hundreds of proteins to activate and propagate a DNA damage response (DDR) (Kitagawa & Kastan, 2005). One such phosphorylation target is histone H2AX. This phosphorylation event forms γH2AX, an important marker of DNA damage (Fig. 1.3A). The DDR signal transducer MDC1 binds to γH2AX and recruits the E3 ligase RNF8. RNF8 is ubiquitinates H2A at lysine (K) 13 or 15. This ubiquitination event (hereafter abbreviated as H2AK15Ub) is unique to DNA damage signaling. It facilitates recruitment of another E3 ligase RNF168, which then extends and amplifies H2A ubiquitination (Huen et al., 2007; Mailand et al., 2007; Mattiroli et al., 2012; Wang & Elledge, 2007). Alternatively, it may be that RNF8 ubiquitinates other targets during DDR that recruit RNF168, and it is the latter ligase that is responsible for H2A ubiquitination. The precise dynamics of H2A ubiquitination in response to DNA damage are still an ongoing area of investigation. Regardless H2A must be ubiquitinated at K15 for the recruitment of downstream DDR factors like p53 binding protein 1 (53BP1).

53BP1 is a vital component of NHEJ-mediated DSB repair. It binds to both γH2AX and H2AK15Ub to localize to the site of a break and competes with HR regulator BRCA1 to direct repair towards NHEJ by suppressing the DNA end resection necessary for HR-mediated repair. 53BP1 contains several domains responsible for binding other proteins in DDR to facilitate a variety of functions. The protein-binding domains breast cancer carboxy-terminal (BRCT), ubiquitin-dependent recruitment (UDR), and Tudor bind to multiple different chromatin marks at sites of DSBs: γH2AX, H2AK15Ub, and methylated H4K20 (H2K20me2), respectively (Shibata & Jeggo, 2020b). In addition, the BRCT domain is responsible for binding and activating p53, a critical regulator of proliferation and apoptosis. 53BP1 is phosphorylated by ATM (Baldock et al., 2015; Kitagawa & Kastan, 2005; Lee et al., 2010), which allows recruitment of RIF1 to activate the Shieldin complex and inhibit DNA end resection (Shibata & Jeggo, 2020b).

Nucleases including Artemis and MRE11 of the MRN complex process the broken DNA ends by catalyzing removal of nucleotides and revealing microhomologies (Ma et al., 2002; Moshous et al., 2001; Shibata & Jeggo, 2020a). DNA Ligase IV then works in conjunction with XRCC4 or PAXX to repair the DSBs (Chang et al., 2017). Because of nuclease-dependent processing of DNA ends, NHEJ often results in small insertions and deletions of nucleotides. This mechanism contribute to diversity of Ig genes but can be deleterious at other genomic locations.

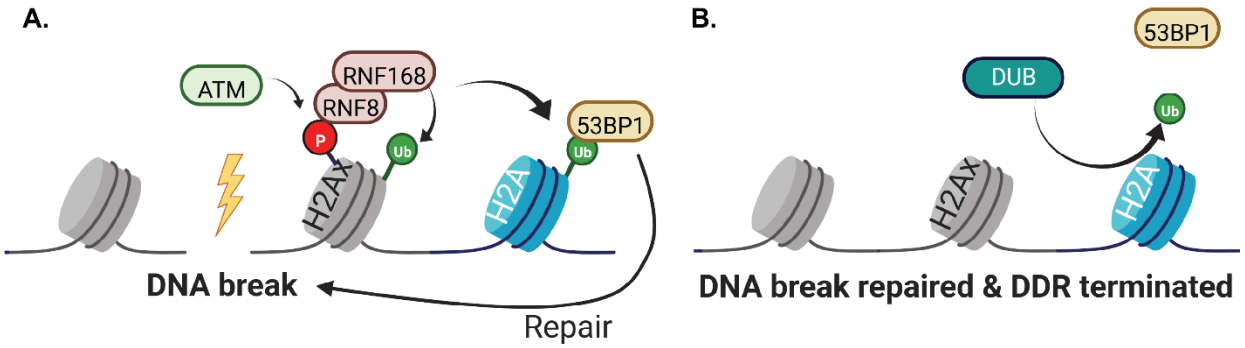

Figure 1.3 DNA damage signaling in V(D)J recombination. **(A)** After generation of a RAG-mediated DSB, ATM is activated and phosphorylates hundreds of proteins including H2AX. E3 ligases RNF8 and RNF168 are recruited to γH2AX and subsequently ubiquitinate H2A to form H2AK15Ub. 53BP1 binds to H2AK15Ub to promote DSB repair through NHEJ. **(B)** After the break is repaired, H2A must be deubiquitinated to resolve 53BP1 foci and turn off DDR signaling. Created in Biorender.com.

1.2.4 Canonical DNA damage responses

The DNA damage response activates a variety of signaling pathways divided amongst repair, cell cycle arrest, and cell death. In addition to previously described NHEJ repair, ATM also phosphorylates KAP1 at serine 824 to promote chromatin relaxation (White et al., 2006). This permits the recruitment of other repair and signaling factors (Ziv et al., 2006).

ATM phosphorylates CHK2 at threonine 68 to promote cell cycle arrest while the damage is repaired (Ahn et al., 2000; Matsuoka et al., 2000; Melchionna et al., 2000). CHK2 is a checkpoint kinase that regulates progression through the cell cycle. Phosphorylation of CHK2 by ATM triggers a cyclin-dependent kinase (CDK) signal cascade resulting in cell cycle arrest in G2. This prevents the cell from entering mitosis and replicating with damaged DNA which can result in deleterious DNA lesions, such as deletions or translocations.

As previously stated, DNA damage can also activate cell death pathways through 53BP1 focus formation and activation of p53. 53BP1 activates p53 through association with the DUB USP26, which then deubiquitinates K48-linked poly-Ub from p53 to stabilize its protein expression and activate p53-mediated cellular programs (Cuella-Martin et al., 2016; Olsson et al., 2007; Rizzotto et al., 2021). However, this function of 53BP1 may be independent of its role in NHEJ (Cuella-Martin et al., 2016). p53 activation in response to DNA damage is also mediated through phosphorylation of p53 by kinases ATM and CHK2, among others (Olsson et al., 2007). p53 triggers cell cycle arrest in G1 through expression of p21, a CDK inhibitor. Prolonged p53 signaling eventually triggers cell death through expression of pro-apoptotic proteins including PUMA and BAX (Helmink & Sleckman, 2012).

1.2.5 Non-canonical DNA damage responses

In addition to this canonical response to DNA damage, RAG-mediated DSBs uniquely initiate an additional, developmental signal cascade in developing B cells (Bednarski et al., 2016; Johnston et al., 2023). This non-canonical response has a variety of roles, including suppression of RAG expression, allelic exclusion of the antigen receptor gene to prevent additional recombination, and pro-survival signals to prevent cell death while DSB repair is mediated (Bednarski et al., 2016; Glynn & Bassing, 2022; Helmink & Sleckman, 2012).

The transcription factor NF-κB is activated by ATM in response to RAG-mediated DSBs. Inactive NF-κB is sequestered by inhibitor of NF-κB α (IκBα). After DSB generation and activation of ATM, IκBα is marked for proteasomal degradation, releasing NF-κB which then translocates to the nucleus and activates transcription (Dunphy et al., 2018; Miyamoto, 2011). In response to RAG DSBs, NF-kB coordinates a broad genetic program. One of the gene targets is the pro-survival factor PIM2 (Bednarski et al., 2012). PIM2 counters p53 activity to inhibit apoptotic signaling and also suppresses cytokine signals to support cell cycle arrest. In combination, these functions of PIM2 allow for repeated attempts at *Ig* recombination if initial efforts are unsuccessful.

In addition to canonical NF-κB, non-canonical NF-κB2 (p100) is expressed in response to RAG-mediated DSBs. After DSB generation through RAG, p100 is processed into its transcriptionally active form of p52. p52 translocates to the nucleus and activates other transcriptional programs. NF-κB2 triggers expression of the transcriptional repressor SPIC, which inhibits pre-BCR signaling and promotes allelic exclusion at the antigen receptor gene locus,

preventing generation of multiple simultaneous DSBs in other areas of the antigen receptor gene locus (Bednarski et al., 2016; Soodgupta et al., 2019).

1.3 Ubiquitin in DNA damage responses

Post-translational modification (PTM) of proteins serves a variety of cellular functions including cellular localization, enzymatic activity, and protein-protein interactions. Some of these modifications include phosphorylation, methylation, and ubiquitination. Ubiquitin (Ub) is a small protein of 76 amino acids that is involved in signaling for many cellular processes (Pickart & Eddins, 2004). Addition or removal of ubiquitin to associated proteins serves as a marker for activation, propagation, or suppression of protein activity and cellular processes. Ubiquitin can be ligated from its C-terminal glycine residue to a lysine residue on a target protein, though methionine, serine, or threonine are also occasionally used (Ikeda, 2023). Ubiquitin can be ligated to a target as a single moiety (monoubiquitin), or it can be ligated to itself to form polyubiquitin (poly-Ub) chains with various functions. These chains commonly extend from methionine 1 (M1), lysine 48 (K48), or lysine 63 (K63) of ubiquitin, though other residues of ubiquitin are also known to be ubiquitinated (K6, K11, K27, K29, K33; Fig 1.4). Different poly-Ub chains serve distinct functions. K48-linked poly-Ub chains designate proteins for degradation by the proteasome. M1- and K63-linked chains serve as scaffolds for cellular signal propagation, often in the context of cellular immune responses (Swatek & Komander, 2016). Notably, K27-linked chains have been shown to be ligated and extended from histones in a DNA damage-dependent context (Gatti et al., 2015). Mixed and branched poly-Ub chains have also been observed (Kaiho-Soma et al., 2021; Ohtake et al., 2016). In addition, ubiquitin can be modified by phosphorylation or acetylation

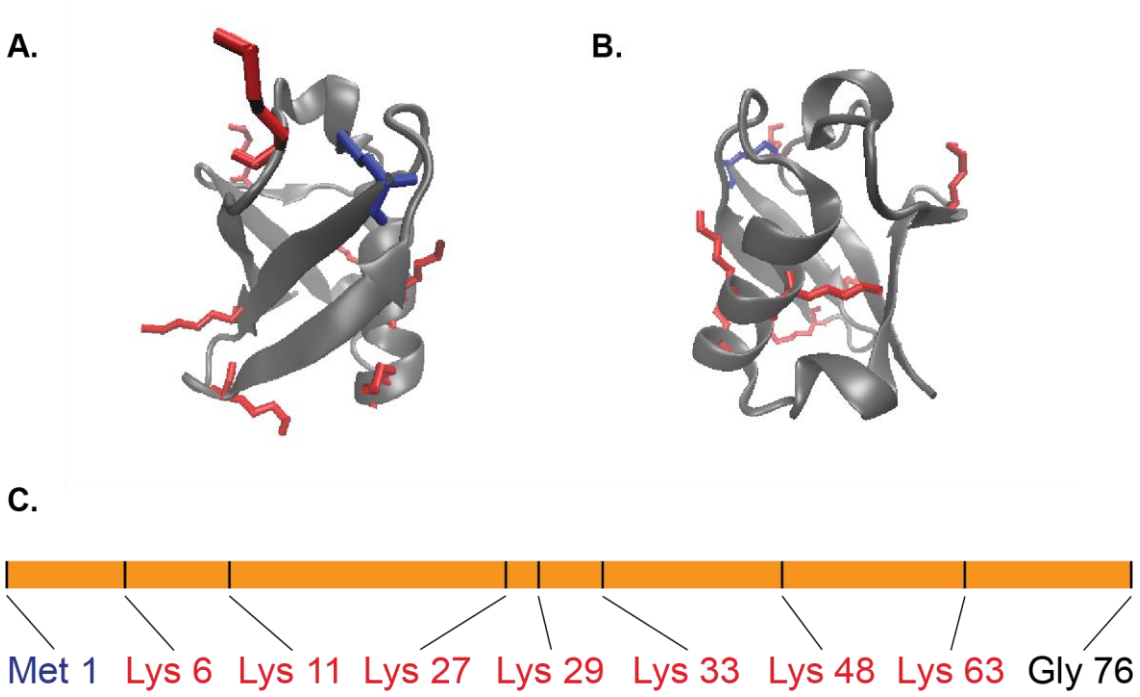

Figure 1.4 Structural diagram of ubiquitin. **(A, B)** Alternate views of the 3D structure of ubiquitin (Huang et al., 2011). Possible residue targets for covalently-linked polyubiquitin chains are labeled in blue (methionine 1) or red (lysines). **(C)** Linear structure of ubiquitin. An E3 ligase connects Ub to its substrate by covalently linking glycine 76 (black) to a substrate, forming an isopeptide bond. Polyubiquitin chains have been demonstrated to extend from methionine 1 (blue), or any of the six lysines (red).

events, but the function of these modifications has largely yet to be determined (Swatek & Komander, 2016).

1.3.1 Ligation of ubiquitin

Ubiquitination is mediated by a group of 3 proteins generally known as E1 activating enzyme, E2 conjugating enzyme, and E3 ligating enzyme. The E1 enzyme activates ubiquitin by conjugating it to a cysteine residue on the E2 enzyme (Ikeda, 2023). The E3 ligase transfers ubiquitin from E2 and ligates it to the target substrate, usually forming an amide bond (Fig 1.5). The combination of E2 and E3 determines substrate specificity for ubiquitination.

There are three types of E3 ligases: really interesting new gene (RING) ligases, homologous to E6-AP carboxyl terminus (HECT) ligases, and RING between RING (RBR) ligases. RING E3 ligases function by transferring Ub from the E2 ligase directly to the substrate (Metzger et al., 2012; Riley et al., 2013; Zheng & Shabek, 2017). Conversely, HECT E3 ligases contain a catalytic cysteine residue that forms a covalent linkage with Ub, removing it from the E2, before linking Ub to the substrate. RBR E3 ligases are a hybrid of the two: they include a canonical RING domain as well as a modified RING domain that contains a catalytic cysteine similar to HECT ligases. RING ligases are the most populous and are involved in every avenue of ubiquitination regulation and signaling (Metzger et al., 2012; Zheng & Shabek, 2017). Though less populous than RING ligases, HECT and RBR ligases are also involved in a variety of cellular processes where ubiquitin is necessary.

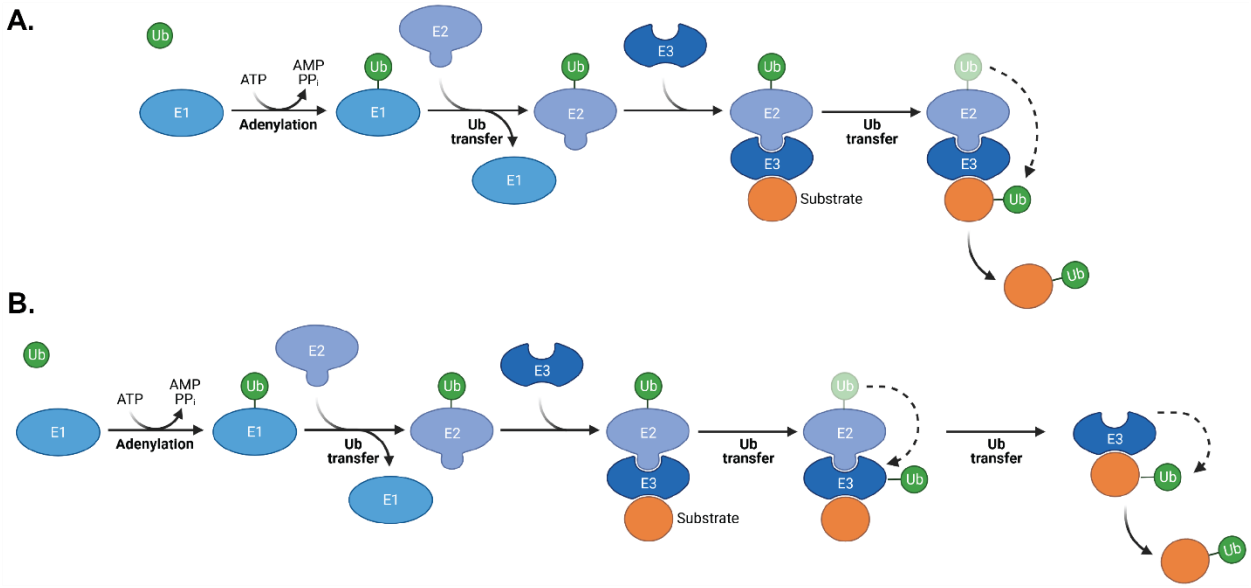

Figure 1.5 Ubiquitination pathways. **(A)** RING E3 ligases transfer Ub from an E2 directly to the substrate. **(B)** HECT E3 ligases form an intermediary step with Ub conjugated to the E3 ligase before transfer to the substrate. Created with Biorender.com.

1.3.2 Ubiquitin removal

Deubiquitinases (DUB) are responsible for removing Ub from target proteins to block or reverse the original ubiquitination signal. There are approximately 100 known DUBs encoded in the human genome, and they are organized into seven families: Ub-specific proteases (USP), ovarian tumor proteases (OTU), Ub C-terminal hydrolases (UCH), Machado-Josephine domain (MJD) proteases, motif interacting with ubiquitin-containing DUB family (MINDY), monocyte chemotactic protein-induced proteins (MCPIP), and MPN domain proteases (Mennerich et al., 2019). DUBs are divided into these families based on sequence and domain similarity. Six of these seven families are cysteine-dependent proteases, but the MPN domain DUBs are instead zinc-dependent metalloproteases.

USPs have the most diverse activity of the seven families. Various USPs have shown activity on monoubiquitinated substrates as well as different poly-Ub chains. OTU DUB activity is specific to poly-Ub chains, though different family members target different types of poly-Ub chains. They are therefore associated with Ub signal modification and attenuation (Clague et al., 2019; Snyder & Silva, 2021). The structure of the UCH domain limits the members of this family to act solely on smaller molecular-weight substrates. Similar to OTU DUBs, MJD DUBs also have multiple Ub-binding domains, though it has yet to be determined if this confers poly-Ub chain specificity. MINDY DUBs are highly specific for K48 poly-Ub chains, and therefore are important in the regulation of proteasomal activity. The MCPIP family is recently discovered, and its members are responsible for regulating innate immunity and inflammation (Jin et al., 2021; Liang et al., 2010).

Unique among known DUBs are the MPN domain-containing proteases. As metalloproteases, their deubiquitinase activity functions distinctly from members of the other six families. Of the 12 members of this family, only 7 contain catalytic activity. These catalytic members contain a conserved catalytic site of the sequence $EX_nHXHX_7SX_2D$. The catalytic residues serine (S), aspartate (D), and both histidines (H) coordinate Zn^{+2} binding and together with the glutamate residue they remove Ub from the target substrate through peptide bond hydrolysis (Pan et al., 2022; Sato et al., 2008). The MPN domain also contains two peptide loops, termed Ins-1 and Ins-2, that determine substrate specificity. The catalytic members of the MPN family display activity on K48- and K63-linked poly-Ub chains as well as monoubiquitinated substrates.

1.3.3 Ubiquitination in gene regulation

Ubiquitination and corresponding deubiquitination of histones, and H2A in particular, are important regulatory processes to control chromatin accessibility, gene expression, and DNA repair. H2A is monoubiquitinated at lysine 119 (H2AK119Ub) by the polycomb repressor complex 1 (PRC1) to silence neighboring gene transcription (Vidal & Starowicz, 2017; Wheaton et al., 2017). PRC1 complexes can contain a variety of different proteins depending on genetic targets (Vidal & Starowicz, 2017). Different PRC1 members can have specificity to different locations in the genome. However, virtually every complex contains an E3 ligase: either RING1A, RING1B (also known as RNF2), or BMI1 (Vidal & Starowicz, 2017). These E3 ligases are specific for H2A at lysine 119. Ubiquitination of H2A at this residue is associated with transcriptional repression.

Other members of the complex regulate protein binding and association with various transcription factors to regulate gene expression (Vidal & Starowicz, 2017; Wang et al., 2004).

PRC1 is recruited to YYI in embryonic stem cells (Lu et al., 2018), or to RUNX1 binding sites in T cells and megakaryocytes (Yu et al., 2012). PRC1 activity is required for maintenance of hematopoietic stem cells (Lu et al., 2018; Rizo et al., 2009). In addition, PRC1 regulates expression of important B cell transcription factors EBF1 and PAX5 (Vidal & Starowicz, 2017). Without PRC1 activity, aberrant *Ebf1* and *Pax5* expression can result in B cell lymphomas.

1.3.4 Ubiquitination in DNA damage responses

Ubiquitin signaling is heavily involved in the activation and propagation of DDR. During activation of DDR, E3 ligases RNF8 and RNF168 are recruited and ubiquitinate H2A to form H2AK15Ub. In addition, PRC1 ubiquitination activity has been observed at the site of DSBs, indicating that H2AK119Ub may also be important for the DDR and DSB repair (Ginjala et al., 2011; Ismail et al., 2010). Significant increases in K48- and K63-linked polyubiquitin chains have also been reported in response to DNA damage, though their substrates have yet to be determined (Elia et al., 2015). Consequently, deubiquitination is required for DDR attenuation and termination. Members of the USP DUB family (USP49, USP51) are known to act on H2AK15Ub, and loss of these DUBs results in persistent 53BP1 foci (Matsui et al., 2022; Z. Wang et al., 2016). BRCC36, an MPN domain protease, is necessary for proper HR-mediated repair; BRCC36 deficiency results in excessive DNA end resection despite DSB repair (Ng et al., 2016). However, current evidence suggests that these are not the only DUBs involved in DSB repair.

1.4 MYSM1: The H2A-DUB

MYSM1 is a deubiquitinase that acts specifically on histone H2A. It contains 3 domains: a SANT domain, SWIRM domain, and an MPN domain (Fig 1.6). MYSM1 also contains an LxxLL motif that mediates protein-protein interactions (Plevin et al., 2005; Savkur & Burris,

2004). The SANT domain is necessary for DNA binding. The SWIRM domain is required for MYSM1 binding to deubiquitinase targets in the cytosol (Panda & Gekara, 2018). The MPN domain coordinates Zn^{2+} binding and catalyzes removal of Ub. In addition, MYSM1 contains several serine-glutamine or threonine-glutamine (SQ/TQ) motifs which are known targets of ATM (Kitagawa & Kastan, 2005; Matsuoka et al., 2007). MYSM1 is highly expressed in hematopoietic cell lineages and lymphocytes in particular, but it is also found at low levels in other tissues.

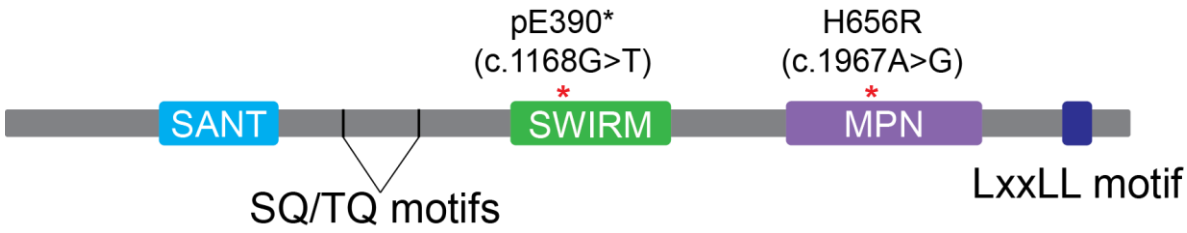

Figure 1.6 Structural diagram of MYSM1. MYSM1 is 828 amino acids long. The SANT domain contains residues 116-167. The SWIRM domain contains residues 372-470. The MPN domain contains residues 577-709. MYSM1 is reported to be phosphorylated by ATM at serine residues 234 and 267. Known MYSM1 mutations in human patients are labeled with red stars. c.1168G>T replaces residue 390 with a premature stop codon, resulting in a block in protein translation (Bahrami et al., 2016). c.1967A>G mutates histidine 656, a catalytic residue of MYSM1 (Le Guen et al., 2015).

1.4.1 MYSM1 in Innate immunity

In addition to its nuclear activity, MYSM1 has been shown to act in the cytoplasm in response to damage- or pathogen-associated molecular pattern recognition receptor (PRR) activity (Panda & Gekara, 2018; Panda et al., 2015; Tian et al., 2020). MYSM1 removes poly-Ub chains to oppose activation of these PRRs and serves to prevent excessive overactivation. Specifically, loss of MYSM1 results in increased expression of pro-inflammatory cytokines including TNFα (Panda & Gekara, 2018; Panda et al., 2015). MYSM1 removes K63-linked poly-Ub chains from TRAF3 & 6 after PRR stimulation (Panda et al., 2015). MYSM1 also removes K63-linked chains from RIP2, as well as M1- and K27-linked chains (Panda & Gekara, 2018). Further, MYSM1 represses the cGAS-STING pathway in innate immunity, likely by deubiquitinated STING (Tian et al., 2020).

1.4.2 MYSM1 in gene expression

MYSM1 acts to deubiquitinate H2A at K119 to oppose PRC1 activity and activate gene transcription (Fiore et al., 2020; Nijnik et al., 2012). MYSM1-dependent activation of protein expression has been evidenced in several developmental lineages, including hematopoiesis and lymphopoiesis.

Loss of MYSM1 results in a defect in hematopoietic maintenance and differentiation, which can be rescued by loss of p53 (Belle et al., 2015). In MYSM1-deficient mouse models, hematopoietic progenitor cells (HPC) are significantly reduced, as well as downstream lymphocytic lineages like B and T cells. In addition, HSC number is significantly increased. Further, MYSM1 deficiency results in increased self-renewal of HSCs and reduced quiescence which could eventually result in exhaustion and depletion of the HSC compartment (Förster et al.,

2015; Wang et al., 2013). MYSM1 activity is known to promote expression of hematopoietic transcription factors including *Gfi1* (Belle & Nijnik, 2014; Wang et al., 2013). Indeed, MYSM1 has been demonstrated to bind directly to promoter and enhancer regions of *Gfi1* and deubiquitinating H2A in these regions to promote transcription. Loss of MYSM1 results in reduced expression of *Gfi1* by maintaining H2AK119Ub at these regulatory regions. Exogenous expression of *Gfi1* can in part recover these defects caused by MYSM1 deficiency, but it is evident that MYSM1 regulates more than Gfi1 alone in HSC maintenance and development. Intriguingly, double-deletion of both MYSM1 and p53 rescues this phenotype (Belle et al., 2015). It is possible that MYSM1 deletion activates pro-apoptotic pathways that are suppressed by deletion of p53 signaling.

In addition, MYSM1 is implicated in T cell development aside from its role in HSC maintenance and *Gfi1* regulation (Förster, Boora, et al., 2017). When MYSM1 was deleted in a T cell-specific context (*MYSM1*$^{fl/fl}$*:CD4-cre*), CD8 T cells were reduced, though minimal impact on CD4 T cells was observed. Remaining CD8 T cells display increased activation of apoptotic and necrotic signaling pathways, as well as increased T cell signaling after cytokine stimulation. Furthermore, loss of MYSM1 results in increased p53 signaling in CD8 T cells in response to irradiation.

Similarly, MYSM1 is also necessary for B cell development in addition to its involvement in HSC maintenance. MYSM1 has also been shown to mediate initial expression of *Ebf1*, an important transcription factor required for B cell development. (Jiang et al., 2011), though MYSM1 does not appear to be required for continued *Ebf1* expression later in development (Förster, Farrington, et al., 2017). EBF1 is required for B cell lineage priming. EBF1 deficiency results in a block of B cell development at the CLP stage (Vilagos et al., 2012; Zandi et al., 2008).

EBF1 is required for expression of canonical B cell markers like B220, and loss of EBF1 blocks IgH D-J rearrangement, the first stage of V(D)J recombination in developing B cells. Furthermore, EBF1 is indicated to regulate RAG1 expression. MYSM1 is observed to bind to the *Ebf1* promoter Ebfα, and MYSM1 deficiency is associated in increased ubiquitinated H2A in this region and corresponding decrease in *Ebf1* expression (Jiang et al., 2011; Vilagos et al., 2012). Intriguingly, MYSM1 deletion does not impact *Ebf1* expression in mature B cells (Förster, Farrington, et al., 2017), indicating that MYSM1 may only be necessary for early *Ebf1* expression. However, MYSM1 deletion still results in genotoxic sensitivity that is not explained by a loss of EBF1 alone. This demonstrates that MYSM1 has other roles to play in addition to regulation of B cell-specific transcription factors.

1.4.3 MYSM1 in DNA damage

MYSM1 has also been implicated to play a role in DNA damage signaling (Bahrami et al., 2016; Förster, Farrington, et al., 2017; Kroeger et al., 2020). MYSM1 deficiency has been shown to yield an increased sensitivity to genotoxic stress (Bahrami et al., 2016). Human patients' B cells display increased γH2AX at baseline. In addition, when exposed to UV irradiation MYSM1-deficient peripheral blood mononuclear cells (PBMC) also display increased γH2AX.

MYSM1 has also been demonstrated to associate with DNA repair proteins in a damage-dependent manner (Kroeger et al., 2020). MYSM1 is recruited to γH2AX foci and associates with HELLS in a DNA damage-dependent manner. HELLS is a chromatin remodeling enzyme involved in end resection during HR-mediated repair, and loss of HELLS results in increased genomic instability after DNA damaging agents (Kollarovic et al., 2020).

MYMS1 is required for B cell development aside from its regulation of *Ebf1* expression (Förster, Farrington, et al., 2017). Mb1-cre deletion of MYSM1 results in a block in B cell development first observed at the pro-B cell stage, and this block is exacerbated at the pre-B cell stage. In addition, increased p53 signaling and annexin V, a marker for apoptosis, is also observed at these stages of development. Notably, it is at the pro-B and pre-B stages of development where cells undergo programmed DSB generation through V(D)J recombination.

MYSM1 may also be a phosphorylation target of ATM (Matsuoka et al., 2007). A proteomics screen demonstrated that MYSM1 is phosphorylated at S234 and S267 in response to DNA damage. Notably, these serines are at SQ/TQ motifs which are known targets for ATM (Kim et al., 1999; O'Neill et al., 2000). MYSM1 is also phosphorylated at other residues in cancer, ESC differentiation, or other contexts (Mayya et al., 2009; Ochoa et al., 2020; Zhou et al., 2013). Post-translational modifications (PTM) of MYSM1 appear to regulate its function in the variety of cellular processes with which it is involved, but this has yet to be studied in depth.

1.4.4 MYSM1 in human disease

Loss of MYSM1 has also been reported in several human patients (Bahrami et al., 2016; Le Guen et al., 2015). Mutations observed in these patients include a premature stop codon (Bahrami et al., 2016) or a point mutation of the catalytic domain (Le Guen et al., 2015) (Fig. 1.6). All mutations resulted in B-cell deficiency and eventual bone marrow failure. In addition, remaining lymphocytes display increased sensitivity to DNA damage, characterized by increased γH2AX.

1.5 Gaps in knowledge

MYSM1 has clearly defined roles in innate immunity and transcriptional regulation, but its function in DNA damage signaling is poorly understood. Studies have demonstrated its association with other proteins involved in DNA damage, as well as damage-dependent PTMs. Furthermore, MYSM1 clearly plays a role in B cell development in addition to early activation of *Ebf1* expression. However, the actual function of MYSM1 in DDR and B cell development, and how it achieves this function, have yet to be determined. Why is MYSM1 recruited to other DDR factors? What effect does MYSM1 have in regulation of DNA damage signaling? What role does MYSM1 play in B cell development outside of EBF1 regulation? The following chapter attempts to answer these questions.

Chapter 2

MYSM1 Attenuates DNA Damage Signals Triggered by Physiologic and Genotoxic Breaks

This chapter was written in collaboration with David O'Leary, Nermina Saucier, Faiz Ahmad, Lynn S. White, Marwan Shinawi, Matthew J. Smith, Roshini Abraham, Megan A. Cooper, Maleewan Kitcharoensakkul, Abby M. Green, and Jeffrey J. Bednarski, and is submitted for publication at the Journal of Allergy and Clinical Immunology

Patients with deleterious variants in MYSM1 have a primary immune deficiency characterized by B cell lymphopenia, hypogammaglobulinemia, and increased sensitivity to genotoxins. MYSM1 is a histone deubiquitinase with established activity in regulating gene expression. MYSM1 also localizes to sites of DNA injury but its function in cellular responses to DNA breaks has not been elucidated. We sought to determine the activity of MYSM1 in regulation of DNA damage responses (DDR) to DNA double-stranded breaks (DSBs) generated during immunoglobulin receptor gene (Ig) recombination and by ionizing radiation. MYSM1-deficient pre-B and non-B cells were used to determine the role of MYSM1 in DSB generation, DSB repair, and termination of DDR.

Genetic testing in a newborn with abnormal screen for severe combined immune deficiency, T cell lymphopenia, and near absence of B cells identified a novel splice variant in MYSM1 that results in very low protein expression. Radiosensitivity testing of the patient's peripheral blood lymphocytes showed increased γH2AX, a marker of DNA damage, in B cells in the absence of irradiation suggesting a role for MYSM1 in response to DSBs generated during Ig recombination. Suppression of MYSM1 in pre-B cells did not alter generation or repair of Ig DSBs. Rather, loss of MYSM1 resulted in persistent DNA damage foci and prolonged DDR signaling. Loss of MYSM1 also led to a protracted DDR in U2OS cells with irradiation-induced DSBs. MYSM1 functions in termination of DNA damage responses independent of DNA break generation and repair.

2.1 Introduction

Inborn errors of immunity provide unique opportunities to investigate the role of identified genes in development and function of the immune system. In this regard, homozygous or compound heterozygous variants in *MYSM1* (Myb-like SWIRM and MPN domains 1) have recently been identified in patients with primary immune deficiency characterized by B cell lymphopenia, hypogammaglobulinemia, defective hematopoiesis, and increased sensitivity to genotoxic agents (Alsultan et al., 2013; Bahrami et al., 2016; Le Guen et al., 2015; Li et al., 2020). Notably, in all patients, B cells are nearly absent while the reduction in T cells is less severe and less common (Alsultan et al., 2013; Bahrami et al., 2016; Le Guen et al., 2015; Li et al., 2020). MYSM1-deficient mice have lymphopenia and bone marrow failure similar to human disease (Förster et al., 2015; Förster, Farrington, et al., 2017; Huo et al., 2018; Jiang et al., 2011; Nijnik et al., 2012). Cell-type specific MYSM1 loss in early B cells blocks development at the pro-B and pre-B cell stages (Förster, Farrington, et al., 2017; Jiang et al., 2011). In contrast, *Mysm1* deletion in mature B cells does not alter numbers or function (Förster, Farrington, et al., 2017). Thus, MYSM1 is critical for early B cell development.

MYSM1 is a histone H2A deubiquitinase (DUB) that was identified as a transcriptional regulator (Vissers et al., 2008; Wang et al., 2004; Zhu et al., 2007). Monoubiquitination of H2A on lysine 119 (K119) represses transcription and MYSM1 catalyzes removal of this ubiquitin moiety to initiate gene expression (Liang et al., 2023; Vissers et al., 2008; Wang et al., 2004; Zhu et al., 2007). In early B cells, MYSM1 promotes expression of EBF1, a critical transcriptional regulator of B cell differentiation (Jiang et al., 2011; Mandel & Grosschedl, 2010; Vilagos et al., 2012; Zandi et al., 2008). However, MYSM1 deficiency results in increased markers of DNA damage and increased sensitivity to genotoxic stress, which is not seen with loss of EBF1 (Bahrami

et al., 2016; Nijnik et al., 2012). Thus, the immune defects in MYSM1 deficiency are not explained solely by altered expression of EBF1.

MYSM1-deficient lymphocytes have higher baseline (no exposure to genotoxins) levels of phosphorylated histone H2AX (γH2AX), a marker of DNA damage, as well as higher p53 levels, and increased cell death after irradiation exposure (Bahrami et al., 2016; Nijnik et al., 2012). Deletion of p53 rescues B cell populations in *MYSM1*-deficient mice (Belle et al., 2015). These findings suggest that activation of cell death downstream of DNA damage may contribute to the lymphopenia and immune deficiency in MYSM1 deficiency (Belle et al., 2015). MYSM1 has been shown to localize to DNA breaks and to interact with ubiquitinated H2A at irradiation-induced DNA damage, but little is known about its activity in DDR (Kroeger et al., 2020; Nishi et al., 2014). MYSM1 associates with DNA replication factors but DDR proteins often have distinct activities at replication forks versus at DSBs (Kroeger et al., 2020; Syed & Tainer, 2018; Tarsounas & Sung, 2020). The potential impact of MYSM1 on DDR, particularly in B cells with DSBs generated during antigen receptor assembly, and its role in immunodeficiency have not been explored.

Developing B cells must induce DSBs at antigen receptor loci and resolve them in a timely fashion in order to survive and mature. This process occurs in pro-B and pre-B cells, the developmental stages impacted by loss of *Mysm1* (Bednarski & Sleckman, 2019; Förster, Farrington, et al., 2017; Jiang et al., 2011). Antigen receptor gene recombination occurs through the generation of DSBs by the RAG endonuclease (comprised of the RAG1 and RAG2 proteins) in the G1-phase of the cell cycle (Alt et al., 2013; Fugmann et al., 2000; Helmink & Sleckman, 2012). RAG DSBs are repaired by nonhomologous end joining to ensure proper gene assembly

and ongoing lymphocyte development (Alt et al., 2013; Helmink & Sleckman, 2012). A highly conserved signaling network coordinates cellular responses to DSBs (Alt et al., 2013; Bednarski & Sleckman, 2019; Ciccia & Elledge, 2010; Helmink & Sleckman, 2012; Matsuoka et al., 2007; Shiloh & Ziv, 2013). The primary signaling protein activated by DSBs in G1-arrested cells is the ATM kinase, which orchestrates chromatin modifications at the site of DNA injury, including phosphorylation of histone H2AX and ubiquitination of H2A (Aquila & Atanassov, 2020; Ciccia & Elledge, 2010; Matsuoka et al., 2007; Shiloh & Ziv, 2013; Uckelmann & Sixma, 2017). In response to DSBs, H2A is ubiquitinated on lysines 13 and 15 (for simplicity, abbreviated as H2AK15Ub) by the ubiquitin ligases RNF8 and RNF168 (Aquila & Atanassov, 2020; Doil et al., 2009; Mailand et al., 2007; Uckelmann & Sixma, 2017; Wilson et al., 2016). H2AK15Ub serves as a binding site for 53BP1, which in turn functions to recruit numerous proteins that coordinate DSB repair and activation of DDR, including stimulation of p53 (Cuella-Martin et al., 2016; Doil et al., 2009; Mailand et al., 2007; Wilson et al., 2016). Following DSB resolution, these protein complexes dissipate resulting in termination of the DNA damage response. If the DSBs are not repaired, DDR foci persist and promote activation of p53 and induction of apoptosis (Ciccia & Elledge, 2010; Meek, 2004; Shiloh & Ziv, 2013). DDR foci are inactivated by deubiquitinases (DUBs), which remove ubiquitin chains from H2A and other targets (Aquila & Atanassov, 2020; Citterio, 2015; Nishi et al., 2014; Uckelmann & Sixma, 2017). In many cell types, the protein USP51 resolves H2AK15Ub and terminates DDR, and homologous proteins (USP3, USP37, USP44, and USP49) may have similar roles (Aquila & Atanassov, 2020; Citterio, 2015; Lancini et al., 2014; Matsui et al., 2022; Mosbech et al., 2013; Nishi et al., 2014; Typas et al., 2015; Z. Wang et al., 2016). However, the DUBs that regulate DDR in developing B cells are not known.

The marked B cell lymphopenia and increased DNA damage observed in MYSM1 deficiency suggest that MYSM1 may function in DDRs during B cell development.

Here we present a patient with a novel deleterious variant in *MYSM1* that results in loss of MYSM1 protein and demonstrates increased DDR signaling in patient B cells, even in the absence of exogenous DNA damaging agents. To investigate the function of MYSM1 in cellular responses to physiologic and genotoxic DNA injury, we suppressed MYSM1 in pre-B cells and non-B cells, respectively. We find that MYSM1 does not function in DSB generation or repair, but, rather, regulates cessation of DDR signaling. Loss of MYSM1 results in prolonged DDR signaling that may be detrimental to lymphocyte development and to cellular responses to genotoxins, more broadly. These findings provide new insights into B cell lymphopoiesis and have important implications for clinical management of patients with *MYSM1* deficiency.

2.2 Methods

2.2.1 Patient information and study approval

Informed consent was obtained from the patient's parents. Demographics and clinical data were collected from the St. Louis Children's Hospital Immunodeficiency Database and the medical record. The database and collection of clinical information were approved by the Institutional Review Board (approval number 201107235) of Washington University School of Medicine.

2.2.2 Radiosensitivity testing

Patient's peripheral blood mononuclear cells (PBMCs) were assessed for radiosensitivity as previously described (Cousin et al., 2018). Briefly, PBMCs were rested overnight at 37°C in 5% CO2 then divided into three aliquots. One was reserved as unirradiated and the other two were

subjected to 2 Gy irradiation. At indicated time points, samples were stained for surface markers and intracellular phosphoproteins. Anti-CD45, anti-CD3 and anti-CD19 were from Beckman Coulter. Anti-NKp46 was from BioLegend. Anti-pATM (Ser1981) and anti-γH2AX (Ser139) were from Thermo Fisher Scientific. Cells were quantified by flow cytometry.

2.2.3 Genetic testing

Chromosome microarray was performed by Washington University Cytogenetics and Molecular Pathology Laboratory. NextGen sequencing with concurrent deletion/duplication testing for a panel of 26 genes associated with SCID was performed by GeneDx®. Whole-exome sequencing on the patient and parents was also performed by GeneDx®.

2.2.4 Cell lines

Rag1$^{-/-}$*:Bcl2* and *Art*$^{-/-}$*:Bcl2* abl pre-B cells were previously described (Bredemeyer et al., 2008; Soodgupta et al., 2019). Wild-type (WT) abl pre-B cells were generated by transduction of bone marrow cells from wild-type mice with a retrovirus expressing the v-abl kinase as previously described (Bredemeyer et al., 2006; Johnston et al., 2022). Abl pre-B cells were cultured in DMEM with 10% FBS, 1% PenStrep, 1% sodium pyruvate, 1% NEAA, 1% L-glutamine, and 0.0004% β-mercaptoethanol. To induce cell cycle arrest and induction of RAG DSBs, abl pre-B cells were treated with 3 μM imatinib (10^6 cells/ml) for indicated times prior to harvesting for genomic DNA, protein, and immunofluorescence (Bredemeyer et al., 2008; Johnston et al., 2022; Soodgupta et al., 2019). U2OS cells were cultured in DMEM with GlutaMAX, 10% FBS, and 1% PenStrep. To generate DSBs, U2OS cells were exposed to 5 or 10 Gy irradiation using a Mark I-30 cesium irradiator. All cell lines were verified by genotyping and confirmed to be free of Mycoplasma.

2.2.5 shRNA and cDNA expression

pMSCV INV-GFP retroviral plasmid was a gift from Barry Sleckman (Bredemeyer et al., 2006). For murine abl pre-B cells, shRNA targeting *Mysm1* (5'-ACAGGAAAATTCTGGGTTAATA-3') was cloned into the MSCV-hCD2-mir30 retroviral vector (Bednarski et al., 2012). For human U2OS cells, shRNA targeting *Mysm1* (5'-ACCAGATGGCTCTTATCGCTTA-3') was cloned into lentiviral pFLRu-U6-YFP-Puro vector. cDNA encoding 5' FLAG tagged MYSM1 or MYSM1$^{\Delta E16}$ was cloned into the pOZ-IRES-hCD25 retroviral vector (Bednarski et al., 2016). Retrovirus and lentivirus were produced in PlatE cells (Cell Biolabs) and 293T cells, respectively, by transfection of the viral plasmids with Lipofectamine 2000 (Life Technologies). For lentivirus, pCMV-VSV-G and pCMV-d8.2R were also included in the transfection (Soodgupta et al., 2019; Stewart et al., 2003). Viral supernatant was collected and pooled from 24-72 hours after transfection. Cells were transduced with unconcentrated virus in media with polybrene (5 μg/ml; Sigma-Aldrich) as previously described (Bednarski et al., 2012). Transduced cells were identified by flow cytometric assessment of hCD2, hCD25 or YFP. Abl pre-B cells expressing hCD2 were sorted using anti-hCD2 magnetic beads (Miltenyi Biotec) on MS columns (Miltenyi Biotec) according to the manufacturer's protocol. U2OS cells transduced with pFLRu vector were selected by culture with puromycin (1 μg/ml) for 1 week (until all untransduced cells died).

2.2.6 Western blot analyses

Western blots were done on whole cell lysates as previously described (Bredemeyer et al., 2008). Anti-phospho-KAP1 antibody (A300-767A) was from Bethyl Laboratories. Total KAP1 antibody was from Genetex (clone N3C2). Anti-PIM2 (clone 1D12) was from Santa Cruz Biotechnology. Anti-FLAG (clone M2) was from Sigma. Anti-GAPDH (clone D16H11), anti-

phospho-CHK2 (polyclonal; 2661), and total CHK2 (polyclonal; 2662) antibodies were from Cell Signaling Technology. Secondary reagents were horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Cell Signaling) or anti-rabbit IgG (Cell Signaling). Westerns were developed with ECL (Pierce) and ECL Prime (Cytiva). ImageJ was used to quantitate Western blots.

2.2.7 RT-PCR

PCR over the break assay was performed as previously described (Johnston et al., 2022). Briefly, genomic DNA was isolated then digested with NEBNext dsDNA Fragmentase (NEB) for 10 minutes followed by PCR Cleanup (QIAGEN) as per manufacturer's instructions before RT-PCR. For mRNA assessment, RNA was isolated using RNeasy (QIAGEN) and reverse transcribed using oligo-dT primers with SuperScriptII (Life Technologies) according to manufacturers' protocol. RT-PCR was performed using Brilliant II SYBR Green (Agilent) and acquired on an MX3000P (Stratagene) or QuantStudio 3 (ThermoFisher). Each reaction was run in triplicate. For PCR over the break analyses, values at targeted sites were normalized to PCR product spanning CD19, a control region of uncut genomic DNA. For mRNA analyses, values were normalized to PCR product of β-actin. Fold change was determined by the ΔΔ cycle threshold method (Johnston et al., 2022). Primer sequences are in Figure 2.1.

Name	Sequence
Jk1 FWD	GCTACCCACTGCTCTGTTCC
Jk1 REV	CCTTGGAGAGTGCCAGAATC
CD19 FWD	TGTCTCCTTCCTCCTCTTTCT
CD19 REV	CTCAACTCAGAACCCAGACTTT
MYSM1 FWD	GGACAAAGGCATGGACACCATC
MYSM1 REV	TTGAAGACAGCCCTTGGAAGAC
B-actin FWD	TCATCACTATTGGCAACGAGCGGTTC
B-actin REV	TACCACCAGACAGCACTGTGTTGGCA

Figure 2.1 Primers used for PCR over the break and RT-qPCR. Jk1 FWD and REV amplify across a RAG target break. CD19 FWD and REV were used as controls for PCR over the break. MYSM1 FWD and REV amplify across human *Mysm1* transcripts to report overexpression of MYSM1. B-actin FWD and REV were used as controls for measuring mRNA transcripts.

2.2.8 Immunofluorescence

Immunofluorescence microscopy was done as previously described (Johnston et al., 2023; Zhao et al., 2018). U2OS cells were grown directly on coverslips. Abl pre-B cells were applied to coverslips at indicated time points using Cell Tak (Corning) at 37˚C for 20 minutes. Abl pre-B cells were then extracted with 0.2% Triton in PBS for 10 minutes on ice, and both cell types were fixed with 3.2% paraformaldehyde. Cells were washed with IF Wash Buffer (PBS, 0.5% NP-40 and 0.02% NaN_3), then blocked with IF Blocking Buffer (10% FBS in IF Wash Buffer) for 30 minutes at room temperature. Slides were incubated with rabbit anti-53BP1 (Novus; 1:500) in IF Blocking Buffer for 1 hour at room temperature. Slides were washed then stained with goat anti-rabbit IgG conjugated with Alexa Fluor 594 (Invitrogen; 1:1000) and Hoechst 33342 (Sigma-Aldrich) for 30 minutes at room temperature followed by sample mounting with Prolong Gold mounting media (Invitrogen). Microscopy was performed on an Olympus fluorescence microscope (BX-53) using an ApoN 60X/1.49 NA oil immersion lens or an UPlanS-Apo 100X/1.4 oil immersion lens and cellSens Dimension software. Raw images were exported into Adobe Photoshop, and for any adjustment in image contrast or brightness, the levels function was applied.

Foci were quantitated using ImageJ. Each image was analyzed using the following pipeline: first, the DAPI channel was loaded into ImageJ and the image scale was set appropriately. The image was then thresholded to cover each nucleus with minimal overlap. The image was converted into black and white using the Make Binary function. Watershed was then applied to separate any nuclei that may have overlapped. The Analyze Particles function was used to create regions outlining each nucleus. Any irregular regions or regions containing more than one nucleus were excluded. Next, on the 53BP1 channel image the Subtract Background function with a rolling-ball radius of 5-15 μm^2 was applied. The Find Maxima function set to Single Points was

used to identify each focus. The prominence was adjusted to identify as many foci as possible without any false positives. On the newly generated image with the foci as single pixels, the nucleus regions were overlayed, and the Measure function returned the raw pixel density of each nucleus region.

2.2.9 V(D)J Reporter assay

GFP expression was measured by flow cytometry prior to imatinib treatment (0 h) and at indicated time points after imatinib treatment. GFP expression was measured by flow cytometry and normalized to 0 h baseline.

2.2.10 Comet assay

Neutral comet assays were performed using CometAssay (Trevigen) according to manufacturer's instructions. Cells were collected at the desired timepoints and resuspended in PBS at 3 x 10^5 cells/ml. Cell suspension was combined with LMAgarose (Trevigen) at a ratio of 1:10, plated onto a comet slide, and dried at 4°C in the dark. While light-protected, slides were immersed in cold lysis buffer solution (Trevigen) for 1 hour, then in cold TBE for 30 minutes. Slides then underwent electrophoresis at 25V in cold TBE for 30 minutes. Slides were then washed in room temperature DNA precipitation solution (1M ammonium acetate in 95% ethanol) and fixed in 70% ethanol for 30 minutes. After fixation, slides were dried overnight, then stained with SYBR gold (S11494, Invitrogen) for 30 minutes. Images were acquired by fluorescence microscope (Leica DFC3000G) and analyzed by OpenComet in ImageJ.

2.2.11 Statistical Analysis

Statistical analyses were done by Student's t-test using Prism (GraphPad Software).

2.3 Results

2.3.1 Novel MYSM1 variant in patient with B cell aplasia.

A 1-week-old black male presented with abnormal newborn screen for severe combined immune deficiency (SCID). Pre-natal and early post-natal period were notable for small for gestation age but otherwise were unremarkable. Detailed clinical history and evaluation are included in Supplemental Information at the end of this chapter. Laboratory evaluation identified nearly absent B cells ($CD19^+$ = 15 cells/µl), low T cells ($CD3^+$ = 847 cells/µl), and normal NK cells (Fig. 2.2A). Of the total $CD4^+$ T cells, 60% expressed naïve marker CD45RA consistent with mild impairment in thymic function. T cell receptor excision circle testing was low at 1,010 copies per 10^6 $CD3^+$ cells (normal > 6794). T cell proliferation to phytohemagglutinin was normal. No maternal T cell engraftment was present. IgM was very low (2.3 mg/dL, normal 20-149) and IgG was normal (Fig. 2.2A). The low B and T cells persisted through 3 months of age (Fig. 2.2A). He also developed a progressive normocytic anemia (hemoglobin 6.4 g/dL) with inappropriately low reticulocytosis (3%) and he became red blood cell transfusion dependent. Bone marrow biopsy revealed multilineage hematopoiesis, and immunophenotyping showed normal B cell precursors. Genetic testing by clinical whole exome sequencing identified a homozygous variant in *MYSM1* (NM-001085487.2, c.1843-1G>A, IVS15-1 G>A variant) due to paternal uniparental disomy of chromosome 1. This variant is not observed in a large population and is predicted to be pathogenic (Wilson et al., 2016)

2.3.2 MYSM1 variant (c.1843-1G>A) results in aberrant RNA splicing and absent MYSM1 protein

The *MYSM1* (c.1843-1G>A) variant alters the slice acceptor site prior to exon 16. Computational analysis predicted this would result in aberrant slicing that skips exon 16, which encodes the deubiquitinase domain of MYSM1 (Fig. 2.2B). To determine the functional consequences of this novel MYSM1 variant, we first assessed *MYSM1* mRNA in patient peripheral blood mononuclear cells (PBMCs). RT-PCR analysis demonstrated an abnormal *MYSM1* mRNA transcript missing exon 16 (Fig. 2.2B). Sequencing confirmed that patient's *MYSM1* mRNA had splicing of exon 15 to exon 17 with absence of the intervening exon 16 (hereafter termed $MYSM1^{\Delta E16}$).

We next assessed if the $MYSM1^{\Delta E16}$ transcript resulted in expressed MYSM1 protein. Western blot analysis of patient's PBMCs demonstrated loss of high molecular weight MYSM1 protein compared to healthy control PMBCs (Fig. 2.2C). Both control and patient PBMCs demonstrated a lower molecular weight band, which may represent a non-specific band or a MYSM1 variant that could overlap with $MYSM1^{\Delta E16}$ expected size. To distinguish between these possibilities, we retrovirally expressed FLAG-tagged wild-type MYSM1 and $MYSM1^{\Delta E16}$ in 293T cells. $MYSM1^{\Delta E16}$ mRNA was was expressed equivalently to wild-type *MYMS1* mRNA, but both were markedly increased over endogenous *MYSM1* mRNA (Fig. 2.2D). In contrast, $MYSM1^{\Delta E16}$ protein was very low (nearly undetectable) compared to wild-type MYSM1 (Fig. 2.2E). $MYSM1^{\Delta E16}$ protein level is much lower than expected based on mRNA expression, suggesting that the encoded protein is unstable. Collectively, these results demonstrate that the *MYSM1* (c.1843-1G>A) variant results in an abnormally spliced mRNA transcript and absent (or substantially reduced) protein. Thus, clinically, our patient is functionally deficient in MYSM1

A.

	7 days old	2 mon old	Ref. (cells/ul)
T cells	847	491	2500-5500
CD4⁺ T cells	416	181	1600-4000
CD8⁺ T cells	370	207	560-1700
B cells	15	0	200-300
NK cells	554	123	170-1100
CD4⁺CD45RA⁺ T cells	60%	N.D.	n/a

At 7 days old:

IgG	IgM	IgA	IgE
661 mg/dl (nl = 251-1069)	2.3 mg/dl (nl = 20-149)	<7.0 mg/dl (nl = 7-90)	0.2 mg/dl (nl = 0.8-12)

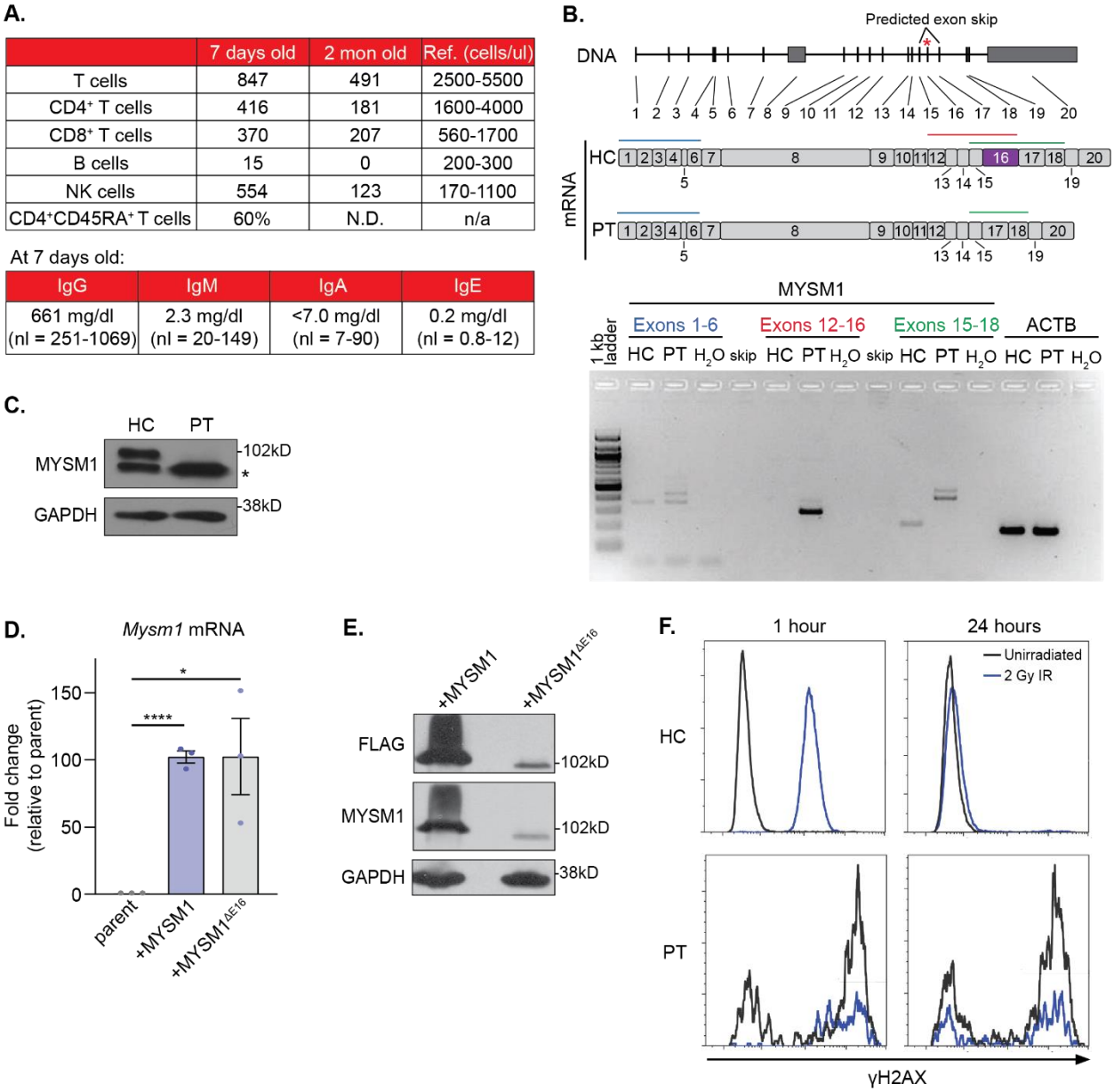

Figure 2.2 Novel MYSM1 splice variant in patient with B cell lymphopenia, hypogammaglobulinemia and increased DNA damage. **(A)** Table of patient's lymphocyte numbers and immunoglobulin values. **(B)** RT-PCR of mRNA isolated from PMBCs of patient (PT) and health control (HC). Exon 1-6 demonstrates mRNA transcription. Exon 12-16 included primer in exon 16. Exon 15-18 shows shorter band with loss of exon 16 in PT sample. Actin-B (ACTB) included as loading control. **(C)** Western blot of analysis of MYSM1 in PBMCs. GAPDH is shown as loading control. * indicates lower molecular band that could represent slice variant or non-specific band. **(D-E)** 293T cells were transfected with vector expressing FLAG-tagged MYSM1 or MYSM1$^{\Delta E16}$. **(D)** Mysm1 mRNA **(E)** Western blot of analysis FLAG-MYSM1. GAPDH is shown as loading control. **(F)** Flow cytometry of γH2AX in unirradiated (black line) B cells from PT and HC and at indicated times after exposure to 2 Gy IR (blue lines). Data in D mean ± SEM from 3 independent experiments, *$p < 0.05$; ****$p < 0.0001$. Data in E are representative of 3 independent experiments.

consistent with other reported patients with MYSM1 mutations (Bahrami et al., 2016; Le Guen et al., 2015) .

2.3.3 Increased DNA damage signaling of naïve MYSM1-deficient B and T cells

Deficiency of MYSM1 in humans and in mouse models has been shown to increase sensitivity to genotoxic stress (Bahrami et al., 2016; Kroeger et al., 2020; Le Guen et al., 2015). To evaluate the functional consequences of the novel MYSM1 variant identified in our patient, we performed radiosensitivity testing on peripheral blood lymphocytes. Phosphorylation of ATM (p-ATM) and H2AX (γH2AX) were measured by flow cytometry in T, B, and NK cells before and after exposure to low dose (2 Gy) irradiation. Patient T cells had broad p-ATM and γH2AX peaks at baseline consistent with activated DNA damage signaling even in the absence of irradiation-induced DNA injury (Fig. 2.2F and Fig. 2.3A, C). Interestingly, nearly all patient B cells had high basal levels of γH2AX, equivalent to levels measured after irradiation. In contrast, there was no evidence of basal DNA damage signaling in the patient's NK cells (Fig. 2.3B, D). Following exposure to irradiation, γH2AX and p-ATM were increased equivalently in both control and patient lymphocytes. Both signals resolved to baseline by 24 hours post-irradiation. Thus, B and T cells are particularly sensitive to loss of MYSM1 with increased DNA damage signaling even in the absence of exogenous DNA damage agents. These lymphocyte-specific findings may reflect aberrant responses to DSBs generated during antigen receptor gene assembly in MYSM1-deficient B and T cells. The markedly higher DNA damage signals in B cells is consistent with the more profound loss of B cells on immune phenotyping and suggests MYSM1 may have unique or additional functions in B cells.

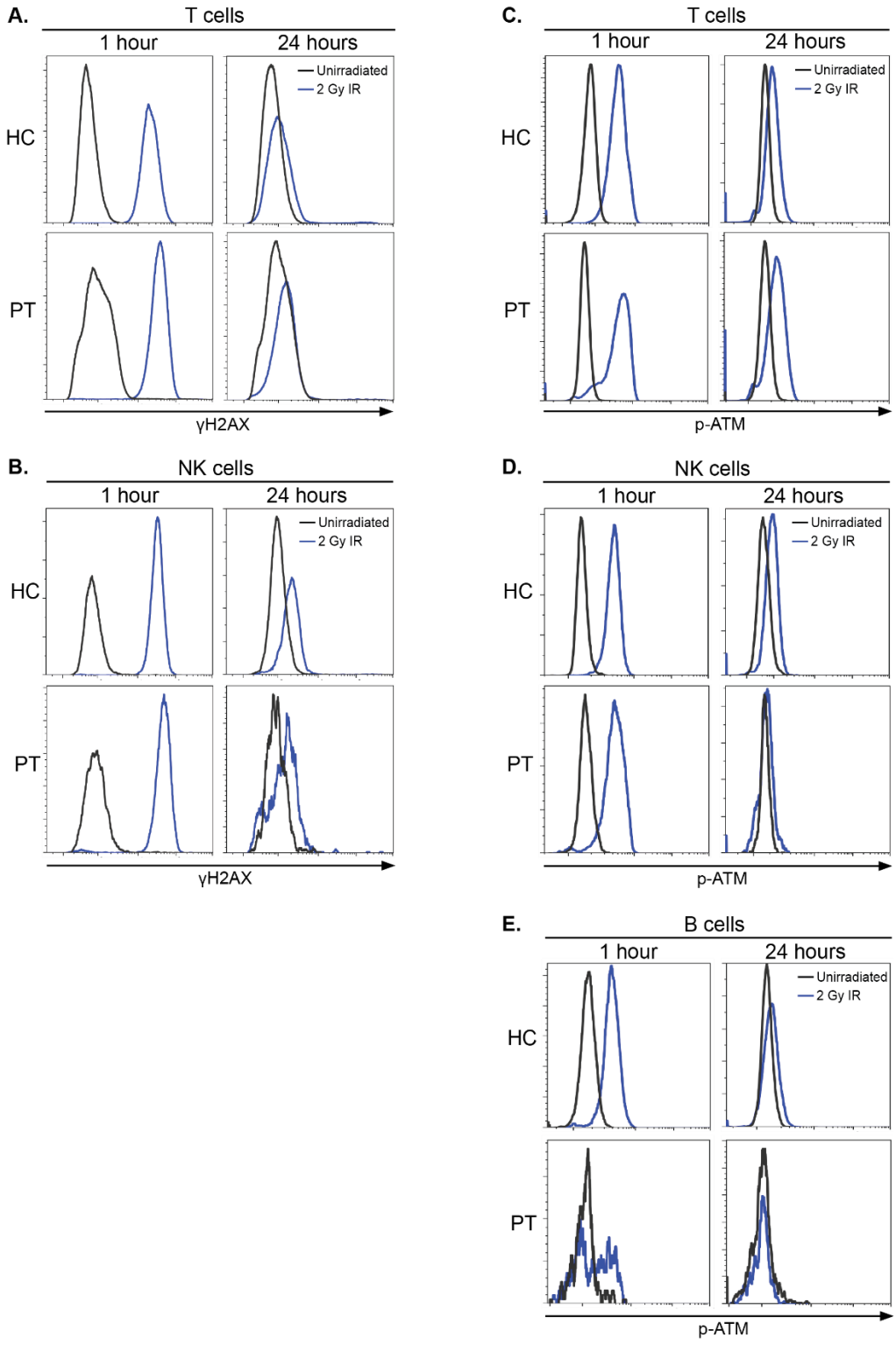

Figure 2.3 DNA damage sensitivity are defects are B cell-specific. Flow cytometry data of either γH2AX **(A, B)** or p-ATM **(C-E)** in T cells **(A, C)**, NK cells **(B, D)**, or B cells **(E)**. Cells were either unirradiated (black line) PT and HC or subject to 2Gy IR (blue lines). Flow cytometry was conducted at indicated times after IR exposure.

2.3.4 Loss of MYSM1 results in persistence of 53BP1 foci in response to DNA damage

Loss of MYSM1 has been shown to result in increased DNA damage signaling in lymphocytes and non-lymphoid cells, but its function in cellular responses to DNA injury has not been characterized. We investigated the role of MYSM1 in DNA damage responses (DDR) to both programmed double-stranded DNA breaks (DSBs) generated during immunoglobulin gene recombination and to genotoxic DSBs. To assess programmed DSBs, we used Abelson-kinase transformed murine pre-B cells (abl pre-B cells) (Bredemeyer et al., 2006; Johnston et al., 2022). Expression of the Abl kinase promotes proliferation and inhibits *IgL* recombination. Inhibition of the Abl kinase with imatinib induces G1 cell cycle arrest, expression of the RAG endonuclease, and recombination of the immunoglobulin light chain (*IgL*) allele (Bredemeyer et al., 2006). *IgL* recombination proceeds through DSB generation which activates DDR signaling, including generation of 53BP1 foci (Bednarski et al., 2012; Bredemeyer et al., 2008). We transduced wild-type (WT) abl pre-B cells with a retrovirus expressing non-targeting shRNA (shNT) or shRNA targeting *Mysm1* (shMysm1) (Fig. 2.4A). Cells were subsequently treated with imatinib to induce *IgL* recombination and associated DDR signaling. 48 hours after imatinib, ~40% of abl pre-B cells accumulated 1-2 53BP1 per cell (Fig. 2.4B, C). As expected, the percentage of cells with 53BP1 foci decreased by 72 hours after imatinib, consistent with DSB repair and resolution of DDR signaling. Suppression of MYSM1 did not affect generation of 53BP1 foci as abl pre-B cells expressing shMysm1 had equivalent 53BP1 foci compared to control cells expressing shNT at 48 hours after imatinib treatment (Fig. 2.4B, C). In contrast, suppression of MYSM1 resulted in a significant increase in 53BP1 foci at 72 hours, indicative of delayed resolution of DNA damage signals (Fig. 2.4B, C).

To determine if MYSM1 also functions in DDR to genotoxic DSBs, U2OS cells were transduced with a lentiviral vector expressing shNT or shMysm1 and exposed to ionizing radiation (IR) (Fig. 2.4D). Both control cells (expressing shNT) and cells with loss of MYSM1 displayed increased 53BP1 foci per cell 2 hours after IR which decreased by 48 hours. Compared to control cells, U2OS cells with loss of MYSM1 demonstrated significantly increased 53BP1 foci per cell at both 2 hours and 48 hours after IR (Fig. 2.4E, F). Thus, similar to findings in pre-B cells with RAG DSBs, loss of MYSM1 results in delayed resolution of 53BP1 foci after IR-induced DSBS. These results demonstrate that MYSM1 functions in responses to both physiologic and genotoxic DNA breaks across different cell types.

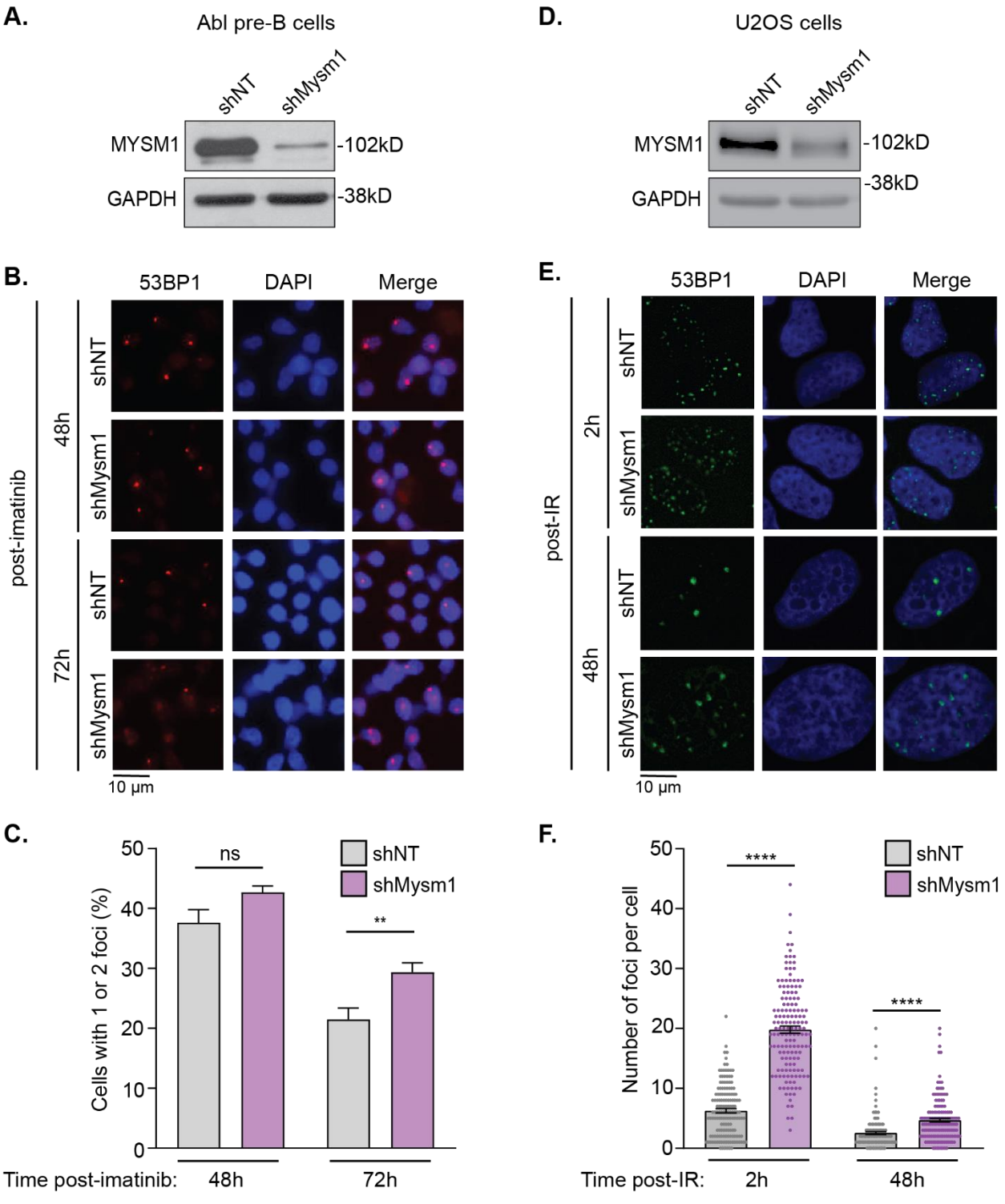
A.
Abl pre-B cells
shNT
shMysm1
MYSM1
-102kD
GAPDH
-38kD
D.
U2OS cells
shNT
shMysm1
MYSM1
-102kD
GAPDH
-38kD
B.
53BP1
DAPI
Merge
post-imatinib
48h
72h
shNT
shMysm1
10 µm
E.
53BP1
DAPI
Merge
post-IR
2h
48h
shNT
shMysm1
10 µm
C.
Cells with 1 or 2 foci (%)
ns
**
shNT
shMysm1
Time post-imatinib:
48h
72h
F.
Number of foci per cell
****
****
shNT
shMysm1
Time post-IR:
2h
48h

Figure 2.4 Loss of MYSM1 results in persistent 53BP1 foci. **(A-C)** Wild-type abl pre-B cells were transduced with retrovirus expressing non-targeting shRNA (shNT) or shRNA targeting murine MYSM1 (shMysm1). **(A)** Western blot of analysis of MYSM1. GAPDH is shown as loading control. **(B)** Representative images of 53BP1 foci at 48 or 72 hours (h) after treatment with imatinib to induce RAG DSBs. Scale bar denotes 10 mm. **(C)** Quantitation of percentage of cells with 1-2 foci per cell in B. Data are mean ± SEM of 3 technical replicates and are representative of 3 independent experiments. (**D**-**F**) U2OS cells were transduced with lentivirus expressing non-targeting shRNA (shNT) or shRNA targeting human MYSM1 (shMysm1). **(D)** Western blot of analysis of MYSM1. GAPDH is shown as loading control. **(E)** Representative images of 53BP1 foci at 2 or 48 h after exposure to 5 Gy irradiation (IR). Scale bar denotes 10 mm. **(F)** Quantitation of number of foci per cell in E. Data are mean ± SEM of 100 cells per condition and timepoint. Results are representative of 3 independent experiments. ns = not significant; **$p < 0.01$; ****$p < 0.0001$.

2.3.5 MYSM1 does not function in generation or repair of DSBs

The impact on 53BP1 foci could be a consequence of MYSM1 function in DSB generation, DSB repair, or regulation of DDR signaling. To resolve this, we measured DSB generation during *IgL* recombination in abl pre-B cells and following irradiation in U2OS cells. We used Artemis-deficient (*Art*$^{-/-}$*:Bcl2*) abl pre-B cells, which generate persistent RAG DSBs at *IgL* as Artemis is required for DSB repair (Soodgupta et al., 2019). Expression of the *Bcl2* transgene in abl pre-B cells supports survival of imatinib-treated, G1-arrested pre-B cells, which permits quantitative evaluation of persistent DSBs. RAG-deficient (*Rag1*$^{-/-}$*:Bcl2*) abl pre-B cells, which do not generate DSBs, were included as a control. Following imatinib treatment, *Art*$^{-/-}$*:Bcl2* abl pre-B cells generated RAG DSBs in ~50% of *Igk* loci (Fig. 2.5A). *Art*$^{-/-}$*:Bcl2* abl pre-B cells with suppression of MYSM1 (expressing shMysm1) had equivalent DSB generation as control cells (expressing shNT; Fig. 2.5A).

To further investigate MYSM1 function in DSB generation and repair, we transduced a repair-sufficient wild-type abl pre-B cell line with a recombination reporter construct containing an inverted GFP cDNA flanked by RAG target sequences. Treatment with imatinib induces RAG DSBs at the target sequences, which are repaired resulting in cDNA inversion and GFP expression (Fig. 2.5B schematic). Expression of GFP from this reporter construct requires both generation and appropriate repair of RAG DSBs. Defects in either process result in reduced GFP expression (Bredemeyer et al., 2006; Lenden Hasse et al., 2017). Pre-B cells expressing shMysm1 had similar GFP expression as control cells expressing shNT (Fig. 2.5B). Together with the above results, these findings demonstrate that loss of MYSM1 in pre-B cells does not affect generation or repair of RAG-mediated DSBs.

We also investigated MYSM1 function in response to IR-induced DSBs. U2OS cells were exposed to 10 Gy IR and DSBs were quantitated by comet assay analysis. Generation of DSBs results in lengthening of the comet tail (measured by olive moment). Subsequent resolution of DSBs is identified by decreased olive moment. 2 hours after IR, U2OS cells have increased olive moment (i.e., DSBs) that is decreased by 48 hours consistent with the expected kinetics of generation and repair, respectively, of IR-induced DSBs (Fig. 2.5C). Suppression of MYSM1 did not alter olive moment at either time timepoint, demonstrating that loss of MYSM1 does not alter generation or repair of genotoxic DSBs (Fig. 2.5C). Together, these results in pre-B cells and U2OS cells indicate that MYSM1 regulates 53BP1 accumulation at DDR foci independent of DSB generation or repair.

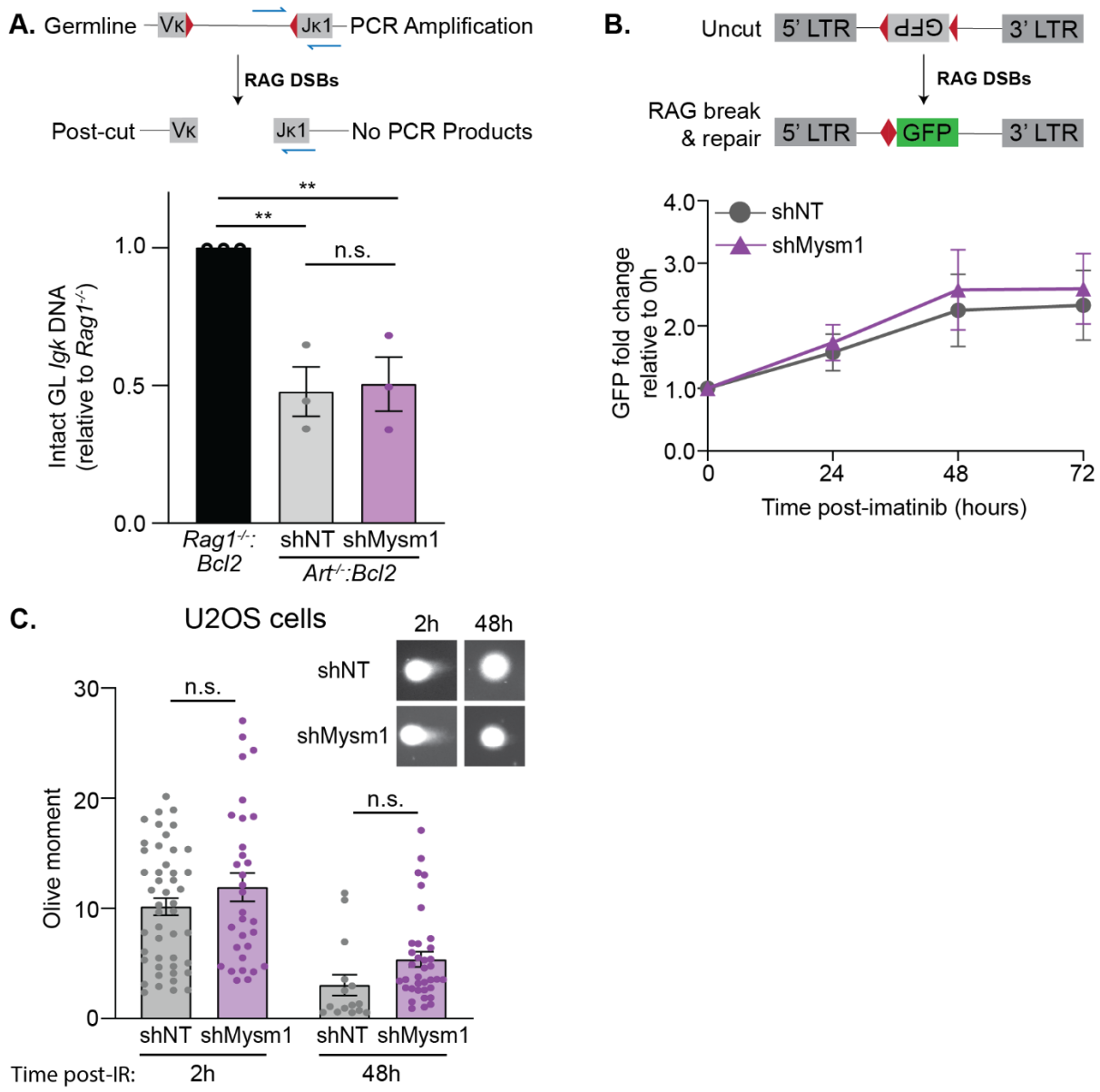

Figure 2.5 MYSM1 does not regulate DSB generation or repair. **(A)** *Art*$^{-/-}$*:Bcl2* abl pre-B cells expressing shNT or shMysm1 were treated with imatinib for 48 h to induce RAG DSBs. DSBs were quantified by qPCR analysis of *IgL* (*Jk1*) genomic DNA. Schematic shows germline locus, unrepaired DSB (post-cut), and primer location. Results are normalized to *Rag1*$^{-/-}$*:Bcl2* abl pre-B cells, which do not generate DSBs and have only germline *IgL* DNA. Loss of germline product is representative of DSB generation. **(B)** Wild-type abl pre-B cells expressing shNT or shMysm1 (as in Fig. 2A) were transduced with a vector encoding a V(D)J reporter construct then treated with imatinib to induce reporter recombination. GFP was measured by flow cytometry at indicated times and normalized to 0 h. Data in A and B are mean ± SEM for 3 independent experiments. **(C)** U2OS cells expressing shNT or shMYSM1 (as in Fig. 2D) were exposed to 10 Gy IR. DSBs were quantitated by comet assay at indicated times. Data are mean ± SEM for olive moment of ≥ 45 cells per condition and timepoint. Inset shows representative images of comet assay. Data are representative of 3 independent experiments. ns = not significant; $^{**}p < 0.01$

2.3.6 Loss of MYSM1 results in persistent downstream DDR signaling

53BP1 coordinates signaling cascades downstream of DSBs and DDR activation (Cuella-Martin et al., 2016; Paiano et al., 2021; Shibata & Jeggo, 2020b). MYSM1 deficiency results in persistence of 53BP1 foci. The continued presence of 53BP1 may stimulate sustained downstream DDR signaling that, in turn, could impact cell fate. To determine whether DDR signaling persists with loss of MYSM1, we assessed DSB-regulated cellular programs in pre-B cells and U2OS cells.

In pre-B cells, RAG DSBs trigger both canonical and noncanonical (developmental) DDR signaling programs (Bednarski et al., 2012; Bednarski et al., 2016; Soodgupta et al., 2019). Canonical DDR signals include phosphorylation of KAP1 whereas noncanonical DDR programs include upregulation of PIM2, a pro-survival factor that promotes continued B cell maturation. Suppression of MYSM1 in wild-type pre-B cells resulted in increased p-KAP1 and expression of PIM2 compared to control pre-B cells (expressing shNT) at both 48 and 72 hours after RAG DSB generation (Fig. 2.6A). In control cells, PIM2 and p-KAP1 decrease at 72 hours compared to 48 hours, similar to the reduction in 53BP1 foci at this timepoint, as expected with DSB repair and termination of DDR signaling (Fig. 2.4B, C and 2.6A). Pre-B cells with loss of MYSM1 also have a reduction in p-KAP1, PIM2, and 53BP1 foci from 48 to 72 hours, but the magnitude of decline is less than in control cells, consistent with persistence of DNA damage signaling (Fig. 2.4B, C and 2.6A). Thus, in the absence of MYSM1 both downstream canonical and non-canonical DDR signaling are increased in magnitude and length of time in pre-B cells undergoing *IgL* recombination.

Similar to pre-B cells, knockdown of MYSM1 resulted in increased phosphorylation of KAP1 and CHK2 (p-CHK2), two key DDR signaling factors, after irradiation in U2OS cells (Fig.

2.6B). Phosphorylation of both proteins was increased as early as 30 minutes after IR and then gradually declines. U2OS cells with loss of MYSM1 also displayed increased p-KAP1 and p-CHK2 at each time point, and both remained higher than in control cells (expressing shNT). As DSBs are repaired, DDR signaling is normally attenuated as evidenced by the decrease of phosphorylated KAP1 and CHK2 by 24 hours after IR in control and MYSM1-knockdown cells (Fig. 2.6B). Notably, phosphorylation of both proteins declines in MYSM1-sufficient and -deficient cells indicating that factors in addition to MYSM1 contribute to resolution of DDR signaling. Despite this redundancy, loss of MYSM1 results in higher magnitude and longer persistence of DDR signaling after RAG- and IR-induced DSBs.

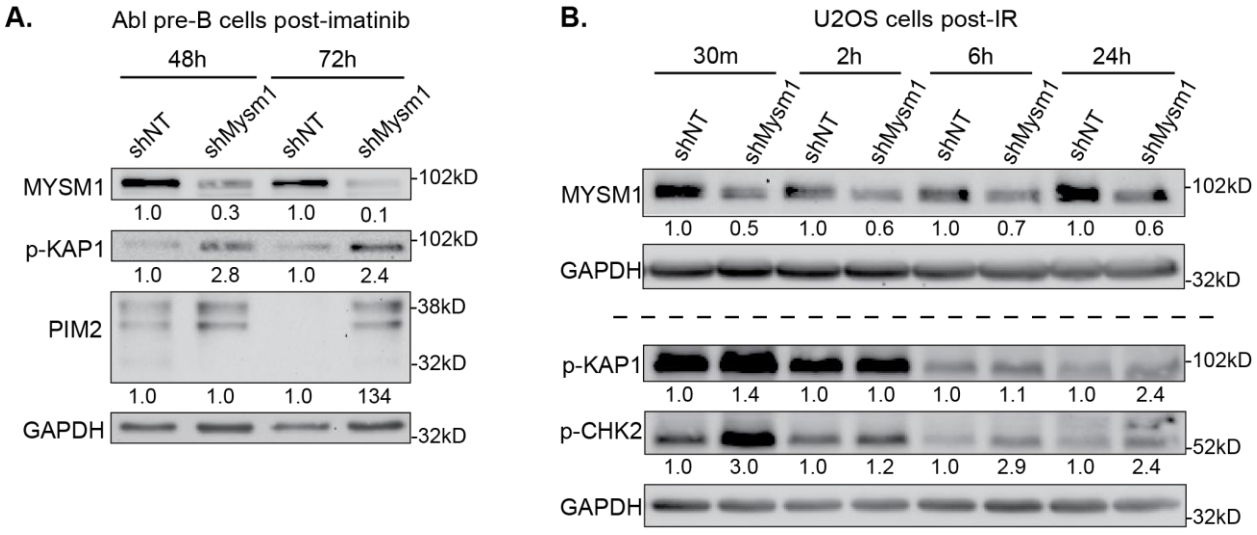

Figure 2.6 Loss of MYSM1 results in prolonged DDR signaling. **(A)** Wild-type abl pre-B cells expressing shNT or shMysm1 (as in Fig. 2.4A) were treated with imatinib to induce RAG DSBs. Western blot analysis of MYSM1, p-KAP1, total KAP1 and PIM2 at indicated times. GAPDH is shown as loading control. Numbers represent band intensity relative to shNT at each timepoint and standardized to loading control. **(B)** U2OS cells expressing shNT or shMYSM1 (as in Fig. 2.4D) were exposed to 10 Gy IR. Western blot analysis of MYSM1, p-KAP1, total KAP1, and p-CHK2 at indicated times. GAPDH is shown as loading control. Numbers represent band intensity relative to shNT at each timepoint and standardized to loading control. Data in both panels are representative of 3 independent experiments.

2.3.7 Increased MYSM1 expression accelerates DDR resolution

The persistence of DDR signaling with reduction in MYSM1 suggests that cellular levels of MYSM1 may regulate kinetics of DDR resolution. To further investigate how MYSM1 levels impact DDR, we transduced U2OS cells with an expression vector containing GFP-tagged MYSM1, which substantially increased MYSM1 protein over endogenous levels in control cells transduced with empty vector (Fig. 2.7C). Compared to controls, cells with increased MYSM1 displayed a marked reduction in 53BP1 foci, p-KAP1, and p-CHK2 by 2 hours after IR (Fig. 2.7A-C). Indeed, many of the cells with high MYSM1 had almost no 53BP1 foci (Fig. 2.7A, B). Increased expression of MYSM1 did not alter generation of DSBs as measured by comet assay (Fig. 2.7D). In combination with the MYSM1 depletion studies, these findings show that MYSM1 levels inversely correlate with 53BP1 retention at DSBs and activation of DDR signaling. Reduction of MYSM1 leads to persistent DDR whereas increased MYSM1 accelerates DDR resolution without impacting kinetics of DSB repair.

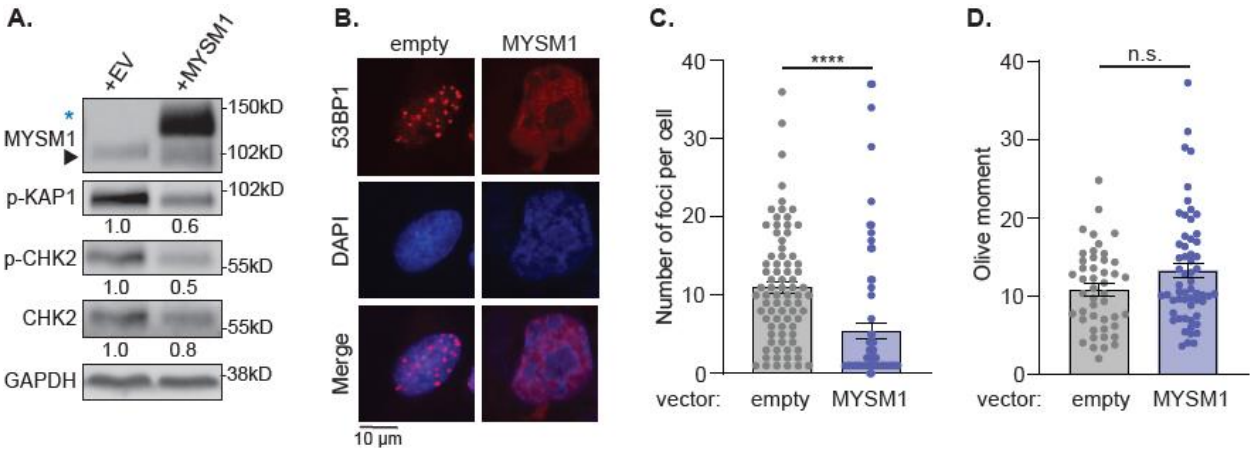

Figure 2.7 Increased expression of MYSM1 suppresses DDR without altering DSB repair. U2OS cells were transfected with empty vector or vector expressing FLAG-MYSM1 then exposed to 5 Gy IR. **(A)** Western blot of MYSM1, p-KAP1, total KAP1, p-CHK2, and total CHK2. GAPDH is shown as loading control. Numbers represent band intensity relative to empty vector control at each timepoint and standardized to loading control. **(B)** Representative images of 53BP1 foci at 2 h after IR. Scale bar denotes 10 mm. **(C)** Quantitation of number of foci per cell in B. Data are mean ± SEM of 100 cells per condition. **(D)** DSBs quantitated by comet assay. Data are mean ± SEM for olive moment of ≥ 45 cells per condition. Data in all panels are representative of 3 independent experiments. ns = not significant; *p < 0.05.

2.4 Discussion

Patients with MYSM1 deficiency have lymphopenia with a disproportionate reduction in B cells, and increased radiosensitivity. Here we show that MYSM1 functions in the termination of DNA damage responses to resolve DDR foci and extinguish DDR signaling after DSB repair. MYSM1 activity in DDR is conserved across both programmed and genotoxic DSBs generated at *Ig* genes during normal B cell development and by exogenous DNA damaging agents, respectively. MYSM1 regulates resolution of 53BP1 retention at DSBs and downstream cellular responses but does not affect DSB generation or repair. Consequently, loss of MYSM1 results in continued DDR signaling without alterations in DSB number or persistence. The activity of MYSM1 in DDR may contribute to the immunophenotype and clinical manifestations of patients with deleterious MYSM1 variants.

Mice with germline deletion of MYSM1 develop bone marrow failure and B cell lymphopenia similar to MYSM1-deficiency in patients. Cell-type specific deletion of MYSM1 in early B cells (at pre-pro B cell stage) results in a marked reduction of pro-B and pre-B cells, the stages where *Ig* gene recombination occurs. MYSM1 was shown to promote expression of *Ebf1*, *Pax5* and *Id2* genes, which are key transcriptional regulators of B cell commitment and differentiation (Förster, Farrington, et al., 2017; Jiang et al., 2011; Wang et al., 2013). Expression of EBF1 rescued some abnormalities in MYSM1-deficient B cells *ex vivo* (Jiang 2011). However, EBF1-deficient mice have a more severe depletion of early B cells at more primitive developmental stages than observed in MYSM1-deficient mice(Gyory et al., 2012; Vilagos et al., 2012; Zandi et al., 2008). Additionally, deletion of MYSM1 at later stages of B cell development does not alter *Ebf1* expression (Förster, Farrington, et al., 2017). Thus, while MYSM1-dependent

regulation of *Ebf1* may affect B cell commitment, MYSM1 has additional functions in regulation of B cell lymphopoiesis.

In addition to altered gene expression, MYSM1-deficient B cells have aberrant DNA damage signals. MYSM1 loss in non-B cells increases sensitivity to DNA damaging agents with increased cell death after ultraviolet or gamma irradiation. In a multidimensional screen of DUBs, MYSM1 was found to localize to DSBs and to regulate DNA repair and the G2/M checkpoint (Nishi et al., 2014). We also find that MYSM1 regulates DDR signaling, including phosphorylation of CHK2, a regulator of cell cycle checkpoint. In contrast, our studies demonstrate that MYSM1 does not regulate DSB generation or repair. This difference may be secondary to different degrees of MYSM1 suppression or distinct MYSM1 activities in different cell types or modes of DNA injury (i.e. irradiation versus physiologic DSBs). We find that MYSM1 has similar functions in responses to physiologic RAG-mediated DSBs and genotoxic IR-induced DSBs in both B and non-B cells, respectively. However, doses of IR, timing of DNA injury in regard to cell cycle state, and cell type can all impact DDR (Syed & Tainer, 2018; Tarsounas & Sung, 2020). Further investigations are needed to characterize the factors that modulate MYSM1 activity in DNA damage.

Histone H2A monoubiquitinated on K119 (H2AK119Ub) is a well-established target of MYSM1 (Nijnik et al., 2012; Wang et al., 2013; Zhu et al., 2007). MYSM1 regulates gene expression through its DUB activity via deubiquitination of H2AK119Ub (Zhu et al., 2007). Mice and humans with mutations in MYSM1 that render it catalytically inactive display a similar phenotype as observed with MYSM1 deficiency, highlighting the role MYSM1 exerts in hematopoiesis and lymphopoiesis through its DUB activity (Liang et al., 2023). Histone H2A is

alternatively ubiquitinated on lysines 13 and 15 at DSBs by the ubiquitin ligases RNF8 and RNF168 (Gatti et al., 2015; Huen et al., 2007; Mailand et al., 2007; Mattiroli et al., 2012). 53BP1 binds H2AK15Ub through its ubiquitin-dependent reader motif and subsequently functions as a transducer to coordinate downstream DDR signaling, including DNA repair, cell cycle arrest and cell death (Fradet-Turcotte et al., 2013). In pre-B cells, 53BP1 prevents DNA end resection to promote joining of RAG DSBs and contributes to regulation of transcriptional programming induced by RAG DSBs (Innes et al., 2020; Paiano et al., 2021; Shibata & Jeggo, 2020b). Following DSB repair, 53BP1 is released from chromatin by DUBs, particularly USP51, which remove K13/15 ubiquitin from H2A (Z. Wang et al., 2016). We find that loss of MYSM1 leads to prolonged retention of 53BP1 at DSBs and continued DDR signaling, which supports that MYSM1 also function in termination of DDR signaling. Interestingly, while 53BP1 foci and DDR signals are prolonged in the absence of MYSM1, both still continue to decline and ultimately resolve, suggesting that other DUBs may cooperate with or compensate for MYSM1 to extinguish DDR. The activity of MYSM1 on H2AK15Ub, the role of its DUB activity, and the function of other DUBs in DDR in early B cells are not known. Additionally, it's not evident whether MYSM1 differentiates between H2AK119Ub and H2AK15Ub or acts equally on both sites. MYSM1 is phosphorylated by ATM in response to DNA injury (Matsuoka et al., 2007). It is conceivable that this modification alters MYSM1 activity or recruitment to chromatin at sites of DNA damage. These mechanisms of MYSM1 and DDR resolution are currently under investigation.

Patients with MYSM1 deficiency have a profound deficiency in B cells that is disproportionate to the reduction in T cells. Our radiosensitivity testing of patient peripheral blood lymphocytes identified DNA damaging signaling in B cells even in the absence of DNA damaging agents. This result is consistent with our finding that MYSM1 regulates DDR to RAG DSBs in B

cells undergoing *Ig* gene recombination. Our results also provide mechanistic context for the reduction in pro-B and pre-B cells in MYSM1-deficient mice. These two developmental stages are when heavy chain and light chain *Ig* genes are rearranged. Thus, they may be more impacted by dysregulation of DDR with MYSM1 loss (Förster, Farrington, et al., 2017). T cells also undergo *Ig* recombination and are reduced in MYSM1 deficiency but not to the same magnitude as B cells. This difference suggests that MYSM1 may have more critical functions in B cells or, alternatively, that B cells and other immune cells have different compensatory mechanisms for managing DDR signaling. MYSM1 does function in genotoxic DNA damage responses but its contribution may be balanced by other DUBs in this context.

Cumulatively, our findings establish that MYSM1 functions in termination of DNA damage responses to both physiologic and genotoxic DSBs. These studies provide new insights into the mechanisms of MYSM1-deficient primary immune deficiency and also highlight need for caution in treatment approaches, as patients will be more sensitive to the chemotherapies used for stem cell transplant.

2.5 Supplemental Information: Patient Clinical Summary

2.5.1 Initial presentation

A 1-week-old healthy, Black male presented with abnormal newborn screen for severe combined immune deficiency (SCID) with a cycle threshold (Ct) value of 45 (reference < 36) on real-time polymerase chain reaction (PCR) for T-cell Receptor Excision Circles (TRECs). Patient was born by spontaneous vaginal delivery to an 18-year-old mother at estimated gestational age of 37 weeks. Maternal pregnancy was complicated by type I diabetes, treated gonococcal infection,

and intrauterine growth retardation. Postnatal course was complicated by low birth weight of 2,140 grams and persistent hypoglycemia. Testing for congenital cytomegalovirus infection was negative. He was discharged on day 4 of life in good health and had no intercurrent infections or illnesses. Family history was notable for neurofibromatosis in paternal aunt but was negative for immunodeficiency, infant death or consanguinity. He has a 2-year-old half-brother who is healthy.

Physical examination revealed an afebrile, small infant with weight 2125 grams, length 41 cm and head circumference 30 cm, all below 3rd percentile for age. No facial dysmorphic features or deformities of extremities were noted. Cardiac exam was without murmur and lungs were clear. He had an exaggerated tonic neck reflex bilaterally and mild hypertonicity of extremities. Remainder of neurological exam was normal.

Brain magnetic resonance imaging (MRI), obtained due to the increased tone, demonstrated nonspecific, scattered T1 hyperintensities within the globus pallidus bilaterally. Ophthalmology exam showed large magnitude infantile exotropia. Hearing test by auditory brain stem response was normal. Bone survey revealed normal bone mineralization with slightly delayed bone age and no deformities. Echocardiogram showed normal systolic function and normal anatomy with mild dilatation of left atrium and ventricle.

Laboratory evaluation on admission (day of life 8) showed white blood cell count of 7000 cells/ml, hemoglobin of 13.9 g/dL, and platelets of 636,000/ml. Absolute lymphocyte count (ALC) was 1500 cells/ml and absolute neutrophil count (ANC) was 4800 cells/ml. Flow cytometric evaluation of immune cell populations identified nearly absent B cells, low T cells, and normal NK cell numbers (Fig. 1A). Of the total $CD4^+$ T cells, 60% expressed CD45RA (Fig. 1A). Naïve $CD31^+CD45RA^+CD4^+$ T cells were slightly reduced at 30% (normal > 64-95%). Repeat TREC

testing was low at 1,010 copies per 10^6 $CD3^+$ cells (normal > 6794). T cell proliferation to phytohemagglutinin was normal (>90%). No maternal T cell engraftment was present. IgM and IgA were low but IgG was normal (Fig. 1A). HIV DNA PCR was negative. Testing of adenosine deaminase and purine nucleoside phosphorylase enzyme activity were normal. Telomere length testing was normal (75^{th} percentile).

2.5.2 Clinical course

The patient was started on monthly intravenous immunoglobulin and prophylactic acyclovir and fluconazole. Prophylactic trimethoprim/sulfamethoxazole (TMP/SMX) was initiated at 4 weeks of age. At 6 weeks of age, he developed normocytic anemia (hemoglobin 6.4 g/dL) with inappropriately low reticulocytosis (3%). Direct and indirect Coombs were negative. Glucose-6-phosphate dehydrogenase level was normal. Bone marrow biopsy revealed multilineage hematopoiesis. He required monthly blood transfusions. TMP/SMX was changed to monthly pentamidine due to a concern of possible bone marrow suppression. Over the subsequent 2 months, T and B cells remained very low (Fig. 1A) and he had three episodes of fever without identified sources. T cell proliferation to phytohemagglutinin repeated at 2 weeks and 4 months of age remained normal.

2.5.3 Hematopoietic stem cell transplant

Due to the persistent T cell lymphopenia and transfusion-dependent anemia, the patient was evaluated for hematopoietic stem cell transplant (HSCT). Patients with MYSM1 deficiency have been successfully transplanted with resultant reconstitution of normal bone marrow and immune function (Bahrami et al., 2016). Our patient proceeded to HSCT at 5 months of age. In light of the radiosensitivity results, a reduced intensity conditioning regimen with low dose

busulfan (target AUC of 40-60 mg x h/L divided over 4 days administered every 6 hours from days -5 to -2), fludarabine (30 mg/m2/day on days -8 to -3) and anti-thymocyte globin (30 mg/kg/day on days -5 to -2) was used followed by infusion of mismatch (5/6) cord blood product. Graft-versus-host disease prophylaxis included mycophenolate mofetil and tacrolimus.

He tolerated conditioning therapy well. On day 5 post-transplant (after infusion of stem cells), he developed fevers with neutropenia (ANC 400 cells/ml). He was empirically treated with cefepime and vancomycin. Due to persistence of fevers, amphotericin B was added. Blood cultures had no growth. Testing for EBV, CMV, HHV-6, adenovirus was negative. On day 9 post-transplant, he developed elevated liver enzymes (AST 374, ALT 532, ALP 197, and LDH 999 units/L), hyperferritinemia (63,622 ng/ml; normal 50-200), ascites, and fluid retention. Abdominal ultrasound showed normal liver without biliary ductal dilatation. Due to concern for venoocclusive disease, defibrotide was started with resultant decrease in liver enzymes. However, he rapidly progressed to acute renal failure with peak creatinine of 1.32 mg/dL (reference 0.1-0.6). On day 13 post-transplant, he was transferred to intensive care unit for hypoxemia requiring intubation and hypertension treated with amlodipine. Chest x-ray demonstrated pulmonary edema. He was initiated on continuous renal replacement therapy. He subsequently was noted to have eye deviation and abnormal limb movement. Electroencephalogram showed diffuse encephalopathy without epileptiform features. Head CT showed no acute intracranial abnormalities. Lumbar puncture was deferred due to unstable conditions. Despite stabilized blood pressure, ventilatory support and correction of electrolytes imbalance, he became profoundly bradycardic and progressed to pulseless electrical activity. Cardiopulmonary resuscitation was initiated but was unsuccessful and the patient died on day 14 post-transplant. A post mortem evaluation was declined by the family and not performed.

Chapter 3

MYSM1 Targets of Activity in DDR

In the previous chapter, we demonstrated that MYSM1 is involved in attenuating DDR in response to both endogenously and exogenously generated DSBs. As previously described, histone H2A is ubiquitinated at K119 to regulate gene expression. However, after DNA lesions H2A is ubiquitinated at K15 to facilitate recruitment of other DNA repair factors including 53BP1. Because MYSM1 is known to act on H2AK119Ub to regulate gene expression, it is plausable that MYSM1 also deubiquitinates H2AK15Ub after break repair. In this manner, MYSM1 would attenuate DDR signaling by promoting the dissolution of 53BP1 foci and downstream signaling.

3.1 Introduction

Ubiquitination is a vital part of DDR necessary for signal propagation and amplification. Significant amounts of K63-linked poly-Ub chains are found at DSBs and used for recruitment of DNA repair factors. K48-linked poly-Ub chains are also found at DSB sites to designate deleterious proteins for degradation (Elia et al., 2015). Monoubiquitin marks are also found on proteins involved in DDR. Histone H2A is mono-ubiquitinated at lysine 13 or 15 (H2AK15Ub) to facilitate recruitment of 53BP1 (Uckelmann & Sixma, 2017; Walser et al., 2020). There is also evidence that K27-linked poly-Ub chains are extended from H2AK15Ub to amplify this recruitment (Gatti et al., 2015). Because all this signaling is transient, corresponding deubiquitination must occur to terminate signaling after DSB repair.

Several deubiquitinases (DUBs) have been implicated in DDR. USP11 deubiquitinates H2AK119Ub as well as histone H2B ubiquitinated at K120 in a DSB-dependent manner (Ting et al., 2019). Loss of USP11 results in persistent ubiquitination of H2B and H2A at K119, but not K13/15, as well as defects in both HR- and NHEJ-mediated repair. These repair defects were

rescued by depletion of E3 ligases known to target H2AK119. Reconstitution with wild-type USP11, but not the catalytically inactive C318S mutant, also rescued repair defects.

USP14 is another DUB implicated in DDR (Sharma & Almasan, 2020; Sharma et al., 2018; X. Wang et al., 2016). USP14 is a member of the USP family and is involved in proteasome regulation. USP14 targets K48-linked poly-Ub chains to prevent protein degradation. Furthermore, USP14 has been implicated in DDR. It is recruited to γH2AX foci after DSB generation and deubiquitinates RNF168 (Sharma et al., 2018). Because ubiquitination of RNF168 is necessary for its recruitment to DSBs, USP14 consequently negatively regulates RNF168 accumulation and therefore inhibits H2AK15Ub and recruitment of 53BP1. Loss of USP14 results in a shift in cell preference of DSB repair methods, resulting in decreased HR and increased NHEJ, characterized by increased RNF168 and 53BP1 foci (Sharma & Almasan, 2020).

USP49 and USP51 are also USP family members, and have been demonstrated to act on H2AK15Ub in response to DNA damage (Matsui et al., 2022; Z. Wang et al., 2016). Loss of USP49 results in persistent ubiquitination of γH2AX, and corresponding overexpression resulted in suppression of γH2AX ubiquitination. Furthermore, USP49 overexpression also suppressed 53BP1 foci formation in response to DNA damage. USP51 deficiency resulted in persistent DDR as measured by 53BP1, BRCA1, and K63 poly-Ub foci. USP51 did not repress RNF168 recruitment to DSBs, but it did suppress ubiquitination of RNF168 target H2A at K13 and 15.

Like MYSM1, BRCC36 is another MPN domain DUB and is an important component of the BRCA1-A complex that regulates DSB repair by HR. BRCC36 removes K63-linked poly-Ub chains at DSBs, and loss of BRCC36 resulted in a block in H-mediated DSB repair. Without BRCC36 activity, aberrant HR repair resulted in excessive DNA end resection (Ng et al., 2016).

Collectively, these demonstrate the critical role of DUBs in modulation of DDR signaling. As the previous chapter demonstrated, MYSM1 is another DUB necessary for DDR attenuation, but its specific deubiquitination target is unknown.

3.2 Methods

3.1.1 Cell Lines

Wild-type (WT) abl pre-B cells were generated as described previously in Chapter 2 (Bredemeyer et al., 2006; Johnston et al., 2022) and cultured in the same media. MYSM1 was knocked down using the previously described shRNA and compared with a non-targeting control sequence (shNT). Cell cycle arrest, RAG expression, and DSB generation were induced by 3 μM imatinib treatment. U2OS cells were transduced with shRNAs, cultured in the same media, and subject to 10Gy IR to induce DSBs as previously described in Chapter 2.

3.1.2 cDNA expression

FLAG-tagged H2A (F-H2A) and FLAG-tagged H2A with K119R mutation (F-H2A^{K119R}) cDNA constructs in the pCDNA3.1 vector were purchased from Addgene (63560 and 63564, respectively). U2OS cells with shMysm1 or shNT control were transiently transfected with either construct in conjunction with Lipofectamine 2000 (10 μl/ml; Invitrogen) then irradiated 24 hours later to generate DSBs.

3.1.3 Immunoprecipitation

Cells were lysed in MCLB buffer (H_2O, 100mM Tris pH 7.5, 1% IGEPAL, 150mM NaCl) and rocked for 30 minutes at 4°C. Lysate was centrifuged at max speed for 20 minutes at 4°C to separate cytosolic and chromatin fractions. Supernatant was removed and saved as the cytosolic fraction. The pellet was then resuspended in MCLB and sonicated to break up nuclei and

chromatin. 10% of this nuclear fraction was saved as input, and the remaining was split between anti-H2A or anti-IgG control beads. Protein A Dynabeads (Invitrogen) were bound to anti-H2A or anti-IgG (control) 24 hours before immunoprecipitation (IP). Beads were washed once with MCLB buffer to remove excess antibody before combining with nuclear lysates. The bead, antibody, and lysate mixture was incubated and rotated at 4°C overnight. Supernatant was collected as the unbound fraction. Beads were then washed 5 times. Bound protein was eluted with elution buffer (H_2O, 1% SDS, 0.84% $NaHCO_3$).

3.1.4 Western blot analyses

Western blots were done on whole cell lysates as previously described (Bredemeyer et al., 2008). Anti-FLAG antibody (clone M2) was from Sigma-Aldrich. Anti-H2A antibody (polyclonal; 2578) was from Cell Signaling. Anti-GAPDH antibody was as described in chapter 2.

3.3 Results

In the previous chapter, MYSM1 was clearly implicated in resolution of the DDR. This was demonstrated by persistence of 53BP1 foci, phosphorylation of KAP1, and expression of PIM2 with loss of MYSM1 after initiation of RAG breaks through imatinib treatment. This persistent DDR signaling was not due to increased DSB generation or defective repair, as MYSM1 did regulate either of these processes. Because MYSM1 has already been described as a known H2A DUB, it is probable that this DUB activity is responsible for DDR attenuation in response to RAG-mediated DSBs.

3.3.1 MYSM1 regulates H2A deubiquitination in pre-B cells with RAG breaks

To investigate MSYM1 activity on ubiquitinated H2A (H2A-Ub), we used wild-type abl pre-B cells transfected with shMysm1 retrovirus to deplete MYSM1 expression as in Chapter 2. Abl pre-B cells were subsequently treated with imatinib to induce cell cycle arrest, expression of RAG endonuclease and resultant activation of DNA damage responses. H2A was immunoprecipitated at 48 hours after imatinib treatment and assessed for ubiquitination. H2A-Ub has an increased molecular weight (17 kD) compared to unmodified H2A (12 kD), and this difference can be visualized by western blot. After imatinib treatment, abl pre-B cells displayed increased H2A-Ub, demonstrating that H2A was ubiquitinated in response to initiation of DNA damage. Cells with depleted MYSM1 exhibited increased H2A-Ub after RAG induction (Fig 3.1A). To confirm this change in H2A-Ub, H2A was IPed from abl pre-B cells with or without MYSM1 knockdown 48 hours after imatinib treatment and assessed for ubiquitination (Fig. 3.1B). Once again we observed increased H2A-Ub with loss of MYSM1, demonstrating that MYSM1 activity on H2A is in part dependent on its activity in DDR signaling.

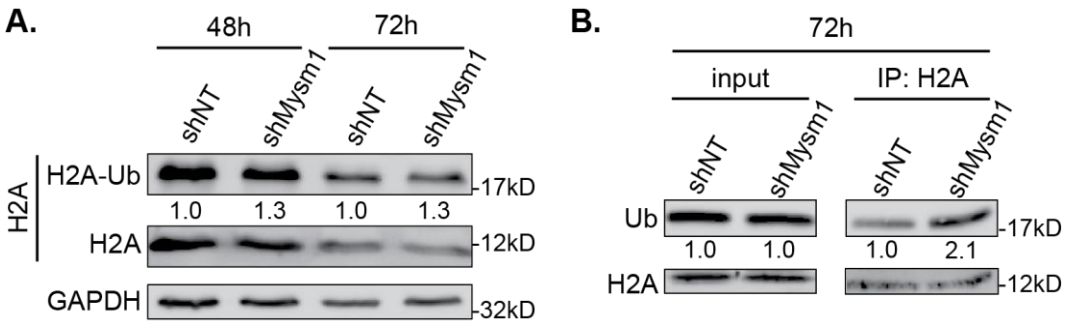

Figure 3.1 MYSM1 deubiquitinates H2A after RAG DSBs. **(A)** Wild-type abl pre-B cells expressing shNT or shMysm1 (as in Fig. 2.4A) were treated with imatinib to induce RAG DSBs. Western blot analysis of H2A at indicated times. GAPDH is shown as loading control. Numbers represent band intensity relative to shNT at each timepoint and standardized to total H2A and loading control. **(B)** Wild-type abl pre-B cells were treated as in **(A)** and H2A was immunoprecipitated at indicated timepoint. Western blot analysis of Ub, with H2A as loading control. Numbers represent band intensity relative to shNT and standardized to loading control.

3.3.2 MYSM1 regulates H2A deubiquitination after IR-induced DSBs

MYSM1 was also shown to be involved in DDR attenuation in response to exogenous DNA damage. Depletion of MYSM1 in irradiated U2OS cells resulted in persistent 53BP1 foci as well as persistent phosphorylation of KAP1 and CHK2, two other markers of DNA damage. Further, overexpression of MYSM1 in irradiated U2OS cells resulted in significant depletion of these markers, despite no impact on DSB repair. These findings demonstrated that MYSM1 regulates DDR termination and suggested this may be through inactivation of H2A ubiquitination. Therefore, H2A ubiquitination in response to IR was measured in this system to determine if MYSM1 achieved DDR attenuation through deubiquitination of H2A.

The impact of MYSM1 on ubiquitinated H2A in response to IR-induced breaks was measured in the same manner as experiments in chapter 2. U2OS cells were transfected with shMysm1 to knockdown MYSM1, or with shNT as a control. Transfected U2OS cell lines were then exposed to 10Gy IR to induce DSBs, and cells were collected for western blot analysis to measure H2A-Ub. Similar to RAG-induced breaks in abl pre-B cells, IR induced increased ubiquitinated H2A in U2OS cells. Loss of MYSM1 resulted in increased ubiquitinated H2A just 30 minutes after irradiation, and this increase persisted up to 24 hours after IR (Fig. 3.2A). This time course parallels the increased DDR signaling with MYSM1 suppression, suggesting a possible mechanistic link. This was further confirmed by immunoprecipitation of H2A 24 hours after IR (Fig. 3.2B), where we observe increased H2A-Ub with loss of MYSM1.

MYSM1 is necessary for deubiquitination of H2A during DDR. However, because this system does not distinguish between H2AK119Ub vs H2AK15Ub, the substrate of MYSM1 activity is not known.

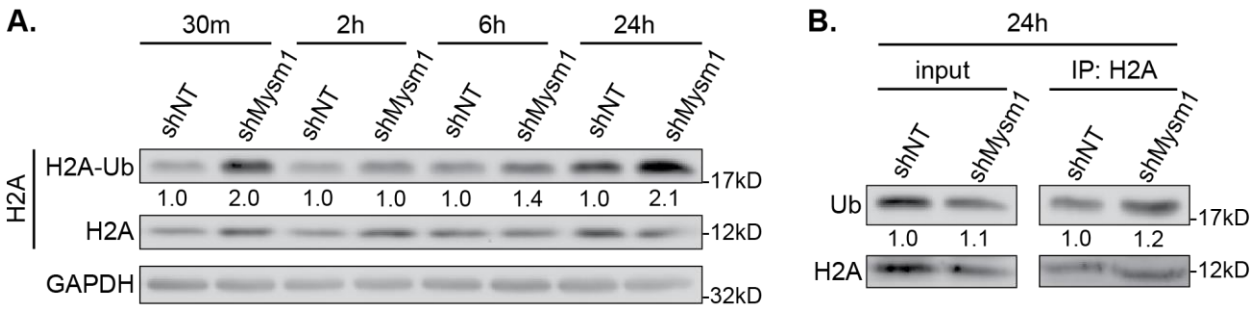

Figure 3.2 MYSM1 deubiquitinates H2A after IR-induced DSBs. **(A)** U2OS cells expressing shNT or shMYSM1 (as in Fig. 2.4D) were exposed to 10 Gy IR. Western blot analysis of H2A at indicated times. GAPDH is shown as loading control. Numbers represent band intensity relative to shNT at each timepoint and standardized to total H2A and loading control. **(B)** U2OS cells were treated as in **(A)** and H2A was immunoprecipitated at the indicated timepoint. Western blot analysis of Ub, with H2A as loading control. Numbers represent band intensity relative to shNT and standardized to loading control.

3.3.3 Does MYSM1 regulate H2AK15Ub?

To investigate whether MYSM1 acts on H2AK119Ub vs H2AK15Ub at DSBs, U2OS cells were transfected with FLAG-tagged wild-type H2A (F-H2A) or an H2A mutant that could not be ubiquitinated at K119 (F-H2A^{K119R}). The F-H2A^{K119R} mutant retains lysines at residues 13 and 15, which can be ubiquitinated by DDR signals but cannot be ubiquitinated on K119, the canonical site for transcription regulation and established target of MYSM1 (Mattiroli et al., 2012). Exogenously expressed F-H2A is expected to be ubiquitinated in a similar manner to native H2A (Mattiroli et al., 2012). Ubiquitination without DNA damage is expected in about 10% of F-H2A at K119. In contrast, F-H2A^{K119R} is expected to only be ubiquitinated at K13/15 in response to DNA damage. If MYSM1 deubiquitinates H2AK15Ub in addition to H2AK119Ub, we would expect to see persistent ubiquitination of F-H2A^{K119R} after IR with loss of MYSM1.

U2OS cells with shMysm1 or shNT were transiently transformed with F-H2A or F-H2A^{K119R} and exposed to 10Gy IR to generate DSBs. Samples were collected 30 minutes after IR to measure H2A ubiquitination by western blotting of FLAG. Unfortunately, despite several attempts, I was not able to visualize ubiquitination of H2A^{K119R} after IR with or without MYSM1 (Fig. 3.3). In order to observe ubiquitinated F-H2A^{K119R} after IR, the exogenously expressed histone must be expressed in sufficient numbers that ubiquitination can be measured through western blotting. It is possible that the construct used did not yield high enough F-H2A^{K119R} to visualize ubiquitination. Alternatively, F-H2A^{K119R} may not have been incorporated into chromatin sufficiently, such that it would not be ubiquitinated after IR. Ongoing studies using IP of endogenous H2A are continuing to investigate function of MYSM1 in regulating H2A ubiquitination during DDR.

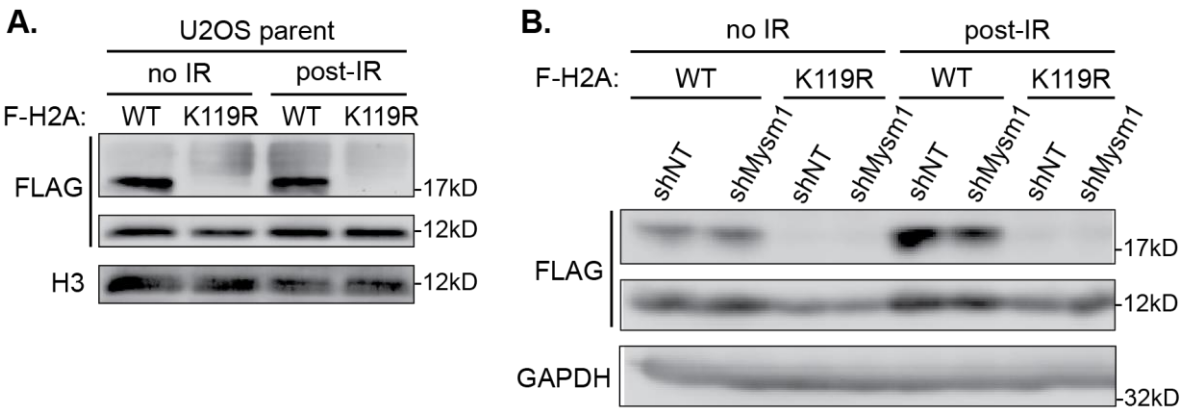

Figure 3.3 H2A is not ubiquitinated at K15 after IR. **(A)** Wild-type U2OS cells were transiently transfected with F-H2A or F-H2A^{K119R} and exposed to 10Gy IR to generate DSBs. Western blot analysis of FLAG at indicated times. H3 was used as a loading control. **(B)** U2OS cells expressing shNT or shMysm1 were transiently transfected with F-H2A or F-H2A^{K119R} and exposed to 10Gy IR to induce DSBs. Western blot analysis of FLAG at indicated times. GAPDH was used as a loading control.

3.4 Discussion

MYSM1 is necessary for termination of DDR as measured by resolution of 53BP1 foci and phosphorylation of DDR proteins. Preliminary results in this Chapter suggest MYSM1 regulates DDR through deubiquitination of H2A. In both pre-B cells and U2OS cells, H2AUb is increased after DSB generation with loss of MYSM1. The increased H2AUb could be ubiquitinated on either K119 or K13/15, as both marks are observed at DSBs. Our studies to date are not able to determine which site on H2A is regulated by MYSM1 at DSBs.

USP11 deubiquitinates H2AK119Ub after DNA damage to promote effective HR-mediated repair. Loss of USP11 results in persistent H2AK119Ub and 53BP1 foci (Ting et al., 2019). Because MYSM1 is also already known to act on H2AK119Ub, it is possible it may function similarly to USP11 in DDR signaling. As described previously, H2AK119Ub is a mark for transcriptional silencing (Vidal & Starowicz, 2017). Its presence at DSBs may be necessary to deactivate transcription machinery during DSB repair. Alternatively, the E3 ligases RNF2 and BMI1 have been implicated in DDR signaling through monoubiquitination of γH2AX at K119. Loss of RNF2 results in decreased γH2AX ubiquitination as well as decreased recruitment of ATM to DSBs (Pan et al., 2011). MYSM1 depletion results in persistent 53BP1 foci (Fig. 2.4B, C, E, F), and it is already known to oppose RNF2-mediated H2A ubiquitination in its function within PRC1. Consequently, MYSM1 may fulfil its function during DDR through H2AK119Ub deubiquitination.

Conversely, USP49 and 51 both target H2AK15Ub to promote resolution of 53BP1 foci. We see that MYSM1 depletion also results in persistent 53BP1 foci, and 53BP1 is known to bind

to H2AK15Ub. Therefore, it is also possible that MYSM1 may function in a similar manner to USP49 or 51.

Ongoing and future studies are continuing to investigate the mechanistic activity of MYSM1 on H2A at DSBs. MYSM1 specificity for H2AK119Ub or H2AK15Ub could be determined through the use of exogenous expression of H2A mutants ($H2A^{K119R}$, $H2A^{K13/15R}$) that would block Ub marks at either K119, or K13 and K15, although my attempts here were as yet unsuccessful. Possible future experiments are discussed in Chapter 4. Current results indicate that MYSM1 likely targets H2AK15Ub in a DNA damage-dependent manner, but more work remains to confirm this hypothesis.

Chapter 4

Implications of Research and Future Directions

The results presented in my thesis have demonstrated for the first time that MYSM1 is involved in attenuation of DDR signaling. These findings support that the abnormal B cell development in mice and patients with loss of MYSM1 may be due to activities of MYSM1 in DNA damage responses. MYSM1 is involved in termination of DDR signaling and regulates resolution of 53BP1 foci and attenuation of downstream signaling proteins. Importantly, these functions of MYSM1 are independent of activity in generation or repair of DSBs. This role of MYSM1 is evident in both pre-B cells in response to RAG-mediated DSBs, as well as in non-B cells in response to IR-induced breaks. As a result, MYSM1 function in DDR signaling could be necessary beyond its clear involvement in B cell development. These findings have implications for treatment of future patients with MYSM1 deficiency as well as for modulating cellular sensitivity to DNA damaging agents.

4.1 Future Directions

MYSM1 catalytic activity has previously been demonstrated on H2AK119Ub to regulate gene expression (Zhu et al., 2007), as well as on several types of poly-Ub chains to regulate innate immunity responses (Panda & Gekara, 2018). Yet, its specific activity on H2AK15Ub has not been observed. Preliminary data indicates that loss of MYSM1 results in persistent ubiquitination of H2A after initiation of DNA damage, but further work is needed to confirm these results. In addition, it remains to be seen if the catalytic activity of MYSM1 is necessary for its involvement in DDR attenuation. Furthermore, my results in conjunction with previous research suggest there may be mechanisms to differentially regulate MYSM1 activity in different cellular processes, i.e. transcriptional regulation, DNA damage responses, and anti-viral responses.

4.1.1 Is MYSM1 DUB activity required for regulation of DDR?

Deubiquitinase activity of MYSM1 has recently been demonstrated to be essential for hematopoietic and lymphocytic development (Liang et al., 2023). An MYSM1 mutant ($MYSM1^{D660N}$) was demonstrated to lack catalytic activity, and replacement of wild-type MYSM1 with $MYSM1^{D660N}$ resulted in a block in hematopoietic lineages. This study affirmed that MYSM1 function in hematopoiesis depends on its DUB activity. However, it remains to be seen if this catalytic activity is necessary for its role in DDR. Wild-type MYSM1 or catalytically inactive $MYSM1^{D660N}$ would be reconstituted in pre-B or U2OS cells and DDR activity could be measured as described in Chapters 2 and 3, namely quantitation of 53BP1 focus and DDR protein resolution as well as H2A ubiquitination. If MYSM1 catalytic activity is necessary for its function in DDR, we would expect to see persistent 53BP1 foci and downstream DDR signaling after reconstitution with $MYSM1^{D660N}$, similar to reconstitution with empty vector.

4.1.2 What are the targets of MYSM1 in regulation of DDR?

The previous chapter suggests that MYSM1 acts on ubiquitinated H2A in a DNA damage-dependent context. H2A is ubiquitinated at lysine 15 after DNA damage, but MYSM1 has been demonstrated to deubiquitinate H2AK119Ub. It is unclear if H2AK15Ub can also serve as a substrate of MYSM1. Because H2AK15Ub is necessary for 53BP1 focus nucleation at DSBs and MYSM1 is involved in resolution of these foci, H2AK15Ub would appear to be a likely target of MYSM1 activity. However, PRC1 is also known to be active at sites of DNA damage, resulting in accumulation of H2AK119Ub. Determining the specific target of MYSM1 DUB activity in DDR would help elucidate the connections between H2A ubiquitination regulation, 53BP1 focus resolution, and transcriptional regulation at DSBs.

As described previously, preliminary research using exogenously expressed H2A lysine-to-arginine mutants has already begun but is inconclusive. FLAG-tagged H2A constructs (F-H2A, F-H2A^{K119R}) do not present clear increases in ubiquitination after DNA damage, so further experimentation is required. Inclusion of a second H2A mutant (F-H2A$^{K13/15R}$) would be useful in examining MYSM1 activity on H2AK119Ub in response to DNA damage. Similar to experiments described in chapter 3, these H2A mutants would be expressed in U2OS cells with or without MYSM1 depletion and ubiquitination would be measured after IR-induced damage. We would expect to see no change in ubiquitination of F-H2A$^{K13/15R}$ after IR, whereas both F-H2A and F-H2A^{K119R} should display increased ubiquitination. MYSM1 has been shown here to deubiquitinate endogenous H2A after DNA damage (Fig. 3.1, 3.2), so comparable results would be expected with exogenous F-H2A expression. If MYSM1 can indeed act on H2AK15Ub after DNA damage, we would expect to observe persistent ubiquitination of F-H2A^{K119R} with MYSM1 depletion.

In addition, MYSM1 deubiquitinase activity on H2A could be measured through chromatin immunoprecipitation (ChIP). DNA adjacent to DSBs would be precipitated with bound proteins, including any H2A mutants present. With loss of MYSM1, we would expect to detect more ubiquitinated H2A. Including the histone mutants F-H2A^{K119R} and F-H2A$^{K13/15R}$ in this assay could potentially measure MYSM1 specificity for H2AK15Ub or H2AK119Ub at DSBs. MYSM1 DUB activity might then be confirmed through acellular experiments with purified MYSM1 and ubiquitinated H2A mutants. In addition, H2A could be immunoprecipitated as in Chapter 3 experiments, and resulting protein would be analyzed by mass spectrometry (MS) to measure ubiquitination at specific lysine residues.

Alternatively, rather than acting on H2A directly, MYSM1 may deubiquitinate other DDR factors. Many other proteins in the DDR are also ubiquitinated to facilitate signal propagation and

amplification. In addition, significant increases in polyubiquitin chains have been detected at DSBs (Elia et al., 2015). Determining MYSM1 activity on other ubiquitinated proteins is more difficult since many of them have yet to be identified, but tandem ubiquitin entity (TUBE) precipitation would be effective in measuring MYSM1 activity on poly-Ub chains involved in DDR. TUBE assays make use of multiple ubiquitin binding domains (UBDs) covalently linked to increase affinity for polyubiquitinated proteins (Kadimisetty et al., 2021). TUBE IP in pre-B or U2OS cells with or without MYSM1 after DSB induction would effectively measure changes in poly-Ub chains after DNA damage, and whether MYSM1 is involved in deubiquitination of these chains. If MYSM1 does not act specifically on H2A-Ub at DSBs, we would expect to see global increase in ubiquitinated proteins with loss of MYSM1. These proteins could subsequently be identified through MS analysis. This approach could also elucidate any changes in DDR signaling that might occur between programmed DSBs during B cell development vs genotoxic DSBs such as those generated by IR.

4.1.3 What are MYSM1 binding partners in DDR?

MYSM1 has a clearly defined role in gene regulation, and here I have demonstrated that it is also important for regulation of DDR. Although MYSM1 appears to act on ubiquitinated H2A in both cases, it is evident that its activity must in turn be regulated to ensure proper deubiquitination at the site of DSBs.

MYSM1 function in DDR may also depend on its association with other DDR proteins. In addition to its catalytic MPN domain, MYSM1 contains a SWIRM domain and an LxxLL motif, both of which can mediate protein-protein interactions (Panda & Gekara, 2018; Plevin et al., 2005; Savkur & Burris, 2004). The SWIRM domain of MYSM1 has already been demonstrated to

facilitate protein interactions in the cytosol during innate immunity (Panda & Gekara, 2018). MYSM1 also contains a SANT domain near its N terminus that is necessary for its activity on H2AK119R (Yoneyama et al., 2007; Zhu et al., 2007). It has yet to be determined if any of these domains are necessary for MYSM1 function in DDR.

MYSM1 is known to associate with various transcription factors (Fiore et al., 2020; Jiang et al., 2011), as well as some proteins associated with DNA damage (Kroeger et al., 2020). Co-immunoprecipitation (co-IP) of MYSM1 has revealed association with HELLS, an important component for DNA repair by HR (Kollarovic et al., 2020). HELLS is a chromatin remodeling protein responsible for promoting HR through DNA end resection factor CtIP.

Further, previous work by a lab member has revealed MYSM1 as a possible binding partner of RAG. These associations could be responsible for regulating the recruitment or activity of MYSM1 at the site of DSBs. Alternatively, MYSM1 could be required for effective function of associated DDR proteins. Elucidating this network in a DNA damage-dependent context would help inform the specific mechanisms for the resolution of DNA damage signaling.

A first approach would include generation of MYSM1 mutants that delete each of these domains (SANT, SWIRM, LxxLL) individually. These mutants, in addition to wild-type MYSM1, would then be expressed in pre-B and U2OS cells with or without induction of DNA damage. Co-IP of these MYSM1 variants would then identify binding partners of MYSM1 that depend on each domain, both dependent and independent of DNA damage. These results could further be confirmed by expressing each individual domain of MYSM1 and repeating co-IPs to determine which binding partners are dependent on specific domains of MYSM1, or if any partners require multiple domains for effective association.

4.1.4 Is MYSM1 activity in DDR regulated by phosphorylation?

The ATM kinase is of vital importance in the activation and propagation of DDR signaling, and it phosphorylates hundreds of proteins to achieve this. Notable ATM targets include H2AX, KAP1, and CHK2. At least two residues (S234, S267) of MYSM1 have been reported to be phosphorylated by ATM in response to DNA damage (Matsuoka et al., 2007), but it is unclear if this phosphorylation is necessary for its role in DDR. Protein phosphorylation is important for regulating both enzyme activity and protein binding. Phosphorylation of MYSM1 could be important for its localization to the sight of DSBs. Alternatively, it may be necessary to enable MYSM1 to act on its substrate during DDR signaling.

Phosphorylation of MYSM1 in response to DNA damage would first be confirmed by a combination of immunoprecipitation (IP) of MYSM1. Antibodies specific for phosphorylated targets of ATM (p-SQ/TQ motifs) can be used to probe MYSM1 IP eluates, or alternatively these anti-phospho-SQ/TQ antibodies could be used for IP and subsequent protein eluates probed for MYSM1. However, because these antibodies are not guaranteed to detect all phosphorylation targets of ATM, mass spectrometry (MS) can be used to detect phosphorylation of MYSM1 more precisely. As previously reported, MYSM1 is expected to be phosphorylated at serines 234 and 267 in response to DNA damage (Matsuoka et al., 2007).

To confirm the necessity of MYSM1 phosphorylation in its function in DDR, MYSM1 phosphomutants would be generated and reconstituted in U2OS cells with MYSM1 depletion. Mutation of any differentially phosphorylated residues of MYSM1 to alanine would block phosphorylation of MYSM1 in response to DNA damage. Corresponding DDR function would be measured as previously shown in chapters 2 and 3, and results would demonstrate the necessity of

MYSM1 phosphorylation to achieve its role in DDR. This could be further confirmed through phosphomimetic mutants of MYSM1. The amino acid aspartate has a similar structure to phosphoserine and is a common induced mutation to simulate constitutively phosphorylated serine (Makukhin & Ciulli, 2021). Expression of similar phosphomimetic mutants of MYSM1 in irradiated U2OS cells could result in overactive MYSM1 and premature resolution of DDR, similar to results observed with wild-type MYSM1 overexpression (Fig. 2.7).

4.1.5 Do other deubiquitinases regulate DDR in pre-B cells?

Deubiquitinases other than MYSM1 are known to be involved in DNA damage, including BRCC36, USP11, USP49, USP51, and others (Matsui et al., 2022; Ng et al.; Sharma et al., 2018; Z. Wang et al., 2016), but DDR-specific H2A DUBs are still poorly understood. Many of these DUBs involved in DDR display a preference for either HR- or NHEJ-mediated DSB repair. BRCC36 targets K63-linked poly-Ub to promote effective HR. USP11 also promotes HR-mediated repair through deubiquitination of H2AK119Ub at DSBs. USP49 and 51 appear to promote NHEJ-mediated repair through regulation of H2AK15Ub and 53BP1 focus resolution. The unique characteristics of MYSM1 suggest that it could be involved in either process. Like BRCC36, MYSM1 is also an MPN domain protease. Like USP11, MYSM1 is known to deubiquitinate H2AK119Ub. Furthermore, results shown previously in chapters 2 and 3 indicate that MYSM1 does not act alone in termination of DNA damage signaling. It is possible that MYSM1 may work in conjunction with other DUBs to effectively mediate DDR termination.

A first step to investigating compensatory DUBs could start with a CRISPR screen using guide RNAs (gRNAs) targeting a pool of other known DUBs expressed in cells already missing MYSM1. After DSB induction, cells with loss of both MYSM1 and compensatory DUBs will

display significantly worse survival, whereas DUBs less important for DSB repair and DDR signaling will have minimal impact. To confirm these results, double-knockout or knockdown cell lines would be generated and DDR signaling would be measured as previously described and compared to single-knockout lines. A double-knockout of both MYSM1 and a compensatory DUB in DDR would be expected to display significantly increased and persistent DDR signaling compared to deletion of either DUB on its own.

4.1.6 Does MYSM1 have distinct activities in homologous recombination?

My research on MYSM1 in DNA damage has focused on its role in DDR signaling after repair by NHEJ because this is the primary method used to repair DSBs, and the sole method used to repair DSBs generated by RAG. Homologous recombination (HR) is an alternative mechanism of repair that uses the sister chromatid as a template to repair DSBs in an error-free fashion. This pathway also relies on ubiquitination and corresponding deubiquitination for proper resolution, but it remains to be seen if MYSM1 is a necessary DUB in these contexts. Association with HELLS indicates that MYSM1 may also be a necessary component for DDR modulation during or after HR-mediated repair. BRCC36, a member of the BRCA1-A complex, is another MPN domain protease involved in HR. MYSM1 may serve as a complementary or redundant DUB for BRCC36 during HR-mediated repair. In addition, regulation of H2AK119Ub has been shown to be important for HR.

HR- and NHEJ-reporter assays could be an effective first step to observe the impact MYSM1 might have in these pathways. These reporter assays function similarly to the V(D)J reporter assay described in chapter 2; they contain disrupted GFP cDNA that can only be repaired by HR or NHEJ, respectively (Kim et al., 1999; O'Neill et al., 2000). Effective repair results in

GFP expression that can be measured in a variety of methods. However, one limitation to this method is that I have already demonstrated that MYSM1 does not impact DSB repair. Consequently, we can measure defects in HR through formation and resolution of RPA or RAD51 foci which form on single-stranded DNA and are therefore unique to HR-mediated repair (Cruz et al., 2018; Maréchal & Zou, 2015). Loss of MYSM1 may result in persistent RPA or RAD51 foci after generation of DSBs.

Alternatively, MYSM1 may instead be involved in pathway choice: the decision to accomplish repair through either NHEJ or HR. The mechanisms of DSB repair pathway choice are still poorly understood. Canonically, 53BP1 and BRCA1 are known to compete for recruitment to DSBs, and they each repress binding of their opposite (Bunting et al., 2010; Daley & Sung, 2014; Kakarougkas et al., 2013; Rass et al., 2022). Commitment to formation of 53BP1 or BRCA1 foci subsequently commits repair towards NHEJ or HR, respectively. However, the competition between these proteins is not a purely random process, and regulation is necessary to dictate repair fate. MYSM1, with implied function in either or both of these pathways, could play a pivotal role in this decision between NHEJ and HR. Pathway choice could be investigated by the formation of 53BP1 vs BRCA1 foci at DSBs. Since MYSM1 has already been demonstrated to result in persistent 53BP1 foci, we might expect to observe a decrease in BRCA1 focus formation with loss of MYSM1.

4.2 Clinical relevance: MYSM1 in human health

As described previously, MYSM1 deficiency has been observed in several human patients (Alsultan et al., 2013; Bahrami et al., 2016; Le Guen et al., 2015), and in chapter 2 we report one more. All of these patients presented with defects in hematopoiesis and B cell development, as

well as increased sensitivity to DNA damage. The research presented here supports MYSM1 as an H2A DUB involved in B cell development and DNA damage, and a probable cause of these observed patient defects. However, persistent DDR signaling with loss of MYSM1 has been demonstrated to be involved in more cell types than developing B cells alone. Indeed, it may be that most, if not all tissues with MYSM1 deficiency present prolonged DNA damage signaling, but this defect is most observable in cells that generate DSBs as part of development (i.e. B cells). Genotoxic sensitivity is also observed in patient T cells (Fig. 2.3A, B) but to a significantly lesser degree, indicating presence of compensatory mechanisms. If MYSM1 is indeed involved in DDR signaling pan-cellularly, patients with defects in MYSM1 may be susceptible to more than just defective HSCs and B cells.

4.2.1 Treatment of MYSM1 deficiency

Previously, MYSM1 deficiency has been treated with bone marrow transplants to replace defective HSCs and downstream lineages (Bahrami et al., 2016). A bone marrow transplant is a risky procedure that necessitates ablation of the patient's original bone marrow and suppression of the patient's immune system to prevent rejection of the new donor graft. (Simpson & Dazzi, 2019). Typical approaches use either irradiation or chemotherapy to achieve both hematopoietic and immune suppression. However, this approach could be problematic in MYSM1 deficiency as the patient would display increased sensitivity to IR. Careful monitoring and/or design of new approaches will be needed for transplant of patients with MYSM1 deficiency.

4.2.2 MYSM1 in cancer pathogenesis

If loss of MYSM1 sensitizes other tissues to genotoxins, patients with MYSM1 deficiency could experience increased risk of cancers such as in the breast or lung. In addition, since loss of

p53 has been shown to rescue MYSM1 deficiency in mouse models (Belle et al., 2015), p53 deficiency might coincide more frequently with MYSM1 deficiency in tumorigenic tissue. Treatment with DNA damaging therapies may also result in increased off-target effects in healthy tissue, making them a riskier treatment option. Alternatively, since loss of MYSM1 results in persistent DDR and increased apoptosis, it may be that cancers with MYSM1 deficiency are more susceptible to DNA damage. More research is necessary to define the possible consequences of MYSM1 deficiency in other tissues as well as in cancers.

4.2.3 MYSM1 as a therapeutic target

The variety of cellular processes that involve MYSM1 make it an interesting target of small molecule inhibition or activation. Because of its role in DDR termination and p53 signaling, it may be an attractive target of inhibition to increase sensitivity to DNA damaging agents. MYSM1 inhibition in conjunction with DNA damage would result in prolonged DDR signaling, potentially ending in apoptosis. Antibiotics thiolutin (THL) and holomycin (HOL) have previously been described as MPN domain inhibitors (Lauinger et al., 2017; Pan et al., 2022). These drugs could be particularly effective because they also inhibit BRCC36 activity, another DUB involved in DDR attenuation.

4.3 Conclusions

Collectively, the results presented here demonstrate a novel role for MYSM1 in the function of DNA damage signaling. This adds to its functions in gene expression and innate immunity. MYSM1 deficiency in humans and in mice results in bone marrow defects and more specifically a block in B cell development. This block is likely due to MYSM1 regulation of B cell-specific transcription factors as well as MYSM1 regulation of cellular responses to DSBs

generated during normal B cell development. The function of MYSM1 in DNA damage responses is not B cell-specific. These results have implications for modulation of DDR signaling as well as clinical approaches for the treatment of new patients with MYSM1 deficiency. In addition, MYSM1 could prove to be an effective therapeutic target for some cancers. More research into MYSM1 cellular function is vital to fully explore the potential of these applications.

References

Ahn, J. Y., Schwarz, J. K., Piwnica-Worms, H., & Canman, C. E. (2000). Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. *Cancer Research*, *60*(21), 5934-5936.

Alsultan, A., Shamseldin, H. E., Osman, M. E., Aljabri, M., & Alkuraya, F. S. (2013). MYSM1 is mutated in a family with transient transfusion-dependent anemia, mild thrombocytopenia, and low NK- and B-cell counts. *Blood*, *122*(23), 3844-3845. https://doi.org/10.1182/blood-2013-09-527127

Alt, F. W., Zhang, Y., Meng, F. L., Guo, C., & Schwer, B. (2013). Mechanisms of programmed DNA lesions and genomic instability in the immune system. *Cell*, *152*(3), 417-429. https://doi.org/10.1016/j.cell.2013.01.007

Aquila, L., & Atanassov, B. S. (2020). Regulation of Histone Ubiquitination in Response to DNA Double Strand Breaks. *Cells*, *9*(7), 1699. https://doi.org/10.3390/cells9071699

Bahrami, E., Witzel, M., Racek, T., Puchałka, J., Hollizeck, S., Greif-Kohistani, N., Kotlarz, D., Horny, H. P., Feederle, R., Schmidt, H., Sherkat, R., Steinemann, D., Göhring, G., Schlegelbeger, B., Albert, M. H., Al-Herz, W., & Klein, C. (2016). Myb-like, SWIRM, and MPN domains 1 (MYSM1) deficiency: Genotoxic stress-associated bone marrow failure and developmental aberrations. *Journal of Allergy and Clinical Immunology*, *1*, 1112-1119. https://doi.org/10.1016/j.jaci.2016.10.053

Baldock, Robert A. A., Day, M., Wilkinson, Oliver J. J., Cloney, R., Jeggo, Penelope A. A., Oliver, Antony W. W., Watts, Felicity Z. Z., & Pearl, Laurence H. H. (2015). ATM Localization and Heterochromatin Repair Depend on Direct Interaction of the 53BP1-BRCT2 Domain with γH2AX. *Cell Reports*, *13*(10), 2081-2089. https://doi.org/10.1016/j.celrep.2015.10.074

Bednarski, J. J., Nickless, A., Bhattacharya, D., Amin, R. H., Schlissel, M. S., & Sleckman, B. P. (2012). RAG-induced DNA double-strand breaks signal through Pim2 to promote pre–B cell survival and limit proliferation. *Journal of Experimental Medicine*, *209*(1), 11-17. https://doi.org/10.1084/jem.20112078

Bednarski, J. J., Pandey, R., Schulte, E., White, L. S., Chen, B.-R., Sandoval, G. J., Kohyama, M., Haldar, M., Nickless, A., Trott, A., Cheng, G., Murphy, K. M., Bassing, C. H., Payton, J. E., & Sleckman, B. P. (2016). RAG-mediated DNA double-strand breaks activate a cell type–specific checkpoint to inhibit pre–B cell receptor signals. *The Journal of Experimental Medicine*, *213*(2), 209-223. https://doi.org/10.1084/jem.20151048

Bednarski, J. J., & Sleckman, B. P. (2019). At the intersection of DNA damage and immune responses. *Nature Reviews Immunology*, *19*(4), 231-242. https://doi.org/10.1038/s41577-019-0135-6

Belle, J. I., Langlais, D., Petrov, J. C., Pardo, M., Jones, R. G., Gros, P., & Nijnik, A. (2015). p53 mediates loss of hematopoietic stem cell function and lymphopenia in Mysm1 deficiency. *Blood*, *125*(15), 2344-2348. https://doi.org/10.1182/blood-2014-05-574111

Belle, J. I., & Nijnik, A. (2014). H2A-DUBbing the mammalian epigenome: Expanding frontiers for histone H2A deubiquitinating enzymes in cell biology and physiology. *The International Journal of Biochemistry & Cell Biology*, *50*, 161-174. https://doi.org/10.1016/j.biocel.2014.03.004

Bredemeyer, A. L., Helmink, B. A., Innes, C. L., Calderon, B., McGinnis, L. M., Mahowald, G. K., Gapud, E. J., Walker, L. M., Collins, J. B., Weaver, B. K., Mandik-Nayak, L., Schreiber, R. D., Allen, P. M., May, M. J., Paules, R. S., Bassing, C. H., & Sleckman, B. P. (2008). DNA double-strand breaks activate a multi-functional genetic program in developing lymphocytes. *Nature*, *456*(7223), 819-824. https://doi.org/10.1038/nature07392

Bredemeyer, A. L., Sharma, G. G., Huang, C.-Y., Helmink, B. A., Walker, L. M., Khor, K. C., Nuskey, B., Sullivan, K. E., Pandita, T. K., Bassing, C. H., & Sleckman, B. P. (2006). ATM stabilizes DNA double-strand-break complexes during V(D)J recombination. *Nature*, *442*(7101), 466-470. https://doi.org/10.1038/nature04866

Bunting, S. F., Callén, E., Wong, N., Chen, H.-T., Polato, F., Gunn, A., Bothmer, A., Feldhahn, N., Fernandez-Capetillo, O., Cao, L., Xu, X., Deng, C.-X., Finkel, T., Nussenzweig, M., Stark, J. M., & Nussenzweig, A. (2010). 53BP1 Inhibits Homologous Recombination in Brca1-Deficient Cells by Blocking Resection of DNA Breaks. *Cell*, *141*(2), 243-254. https://doi.org/10.1016/j.cell.2010.03.012

Chang, H. H. Y., Pannunzio, N. R., Adachi, N., & Lieber, M. R. (2017). Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nature Reviews. Molecular Cell Biology*, *18*(8), 495-506. https://doi.org/10.1038/nrm.2017.48

Ciccia, A., & Elledge, S. J. (2010). The DNA damage response: making it safe to play with knives. *Mol Cell*, *40*(2), 179-204. https://doi.org/10.1016/j.molcel.2010.09.019

Citterio, E. (2015). Fine-tuning the ubiquitin code at DNA double-strand breaks: deubiquitinating enzymes at work. *Frontiers in Genetics*, *6*(SEP), 1-16. https://doi.org/10.3389/fgene.2015.00282

Clague, M. J., Urbé, S., & Komander, D. (2019). Breaking the chains: deubiquitylating enzyme specificity begets function. *Nature Reviews Molecular Cell Biology*, *20*(6), 338-352. https://doi.org/10.1038/s41580-019-0099-1

Cousin, M. A., Smith, M. J., Sigafoos, A. N., Jin, J. J., Murphree, M. I., Boczek, N. J., Blackburn, P. R., Oliver, G. R., Aleff, R. A., Clark, K. J., Wieben, E. D., Joshi, A. Y., Pichurin, P. N., Abraham, R. S., & Klee, E. W. (2018). Utility of DNA, RNA, Protein, and Functional Approaches to Solve Cryptic Immunodeficiencies. *Journal of Clinical Immunology*, *38*(3), 307-319. https://doi.org/10.1007/s10875-018-0499-6

Cruz, C., Castroviejo-Bermejo, M., Guteirrez-Enriquez, S., Llop-Guevara, A., Ibrahim, Y. H., Gris-Oliver, A., Bonache, S., Morancho, B., Bruna, A., Rueda, O. M., Lai, Z., Polanska, U. M., Jones, G. N., Kristel, P., de Bustos, L., Guzman, M., Rodriguez, O., Grueso, J., Montalban, G., . . . Serra, V. (2018). RAD51 foci as a functional biomarker of homologous recombination repair and PARP inhibitor resistance in germline BRCA-mutated breast cancer. *Annals of Oncology*, *29*(5), 1203-1210. https://doi.org/10.1093/annonc/mdy099

Cuella-Martin, R., Oliveira, C., Lockstone, E., Helen, Snellenberg, S., Grolmusova, N., & Chapman, R., J. (2016). 53BP1 Integrates DNA Repair and p53-Dependent Cell Fate Decisions via Distinct Mechanisms. *Molecular Cell*, *64*(1), 51-64. https://doi.org/10.1016/j.molcel.2016.08.002

Daley, J. M., & Sung, P. (2014). 53BP1, BRCA1, and the Choice between Recombination and End Joining at DNA Double-Strand Breaks. *Molecular and Cellular Biology*, *34*(8), 1380-1388. https://doi.org/10.1128/mcb.01639-13

Doil, C., Mailand, N., Bekker-Jensen, S., Menard, P., Larsen, D. H., Pepperkok, R., Ellenberg, J., Panier, S., Durocher, D., Bartek, J., Lukas, J., & Lukas, C. (2009). RNF168 Binds and Amplifies Ubiquitin Conjugates on Damaged Chromosomes to Allow Accumulation of Repair Proteins. *Cell*, *136*(3), 435-446. https://doi.org/10.1016/j.cell.2008.12.041

Dunphy, G., Flannery, S. M., Almine, J. F., Connolly, D. J., Paulus, C., Jønsson, K. L., Jakobsen, M. R., Nevels, M. M., Bowie, A. G., & Unterholzner, L. (2018). Non-canonical Activation of the DNA Sensing Adaptor STING by ATM and IFI16 Mediates NF-κB Signaling after Nuclear DNA Damage. *Molecular Cell*, *71*(5), 745-760.e745. https://doi.org/10.1016/j.molcel.2018.07.034

Elia, E. H., Andrew, Boardman, P., Alexander, Wang, C., David, Huttlin, L., Edward, Everley, A., Robert, Dephoure, N., Zhou, C., Koren, I., Gygi, P., Steven, & Elledge, J., Stephen. (2015). Quantitative Proteomic Atlas of Ubiquitination and Acetylation in the DNA Damage Response. *Molecular Cell*, *59*(5), 867-881. https://doi.org/10.1016/j.molcel.2015.05.006

Fiore, A., Liang, Y., Lin, Y. H., Tung, J., Wang, H., Langlais, D., & Nijnik, A. (2020). Deubiquitinase MYSM1 in the Hematopoietic System and beyond: A Current Review. *International Journal of Molecular Sciences*, *21*(8), 3007. https://doi.org/10.3390/ijms21083007

Förster, M., Belle, J. I., Petrov, J. C., Ryder, E. J., Clare, S., & Nijnik, A. (2015). Deubiquitinase MYSM1 Is Essential for Normal Fetal Liver Hematopoiesis and for the Maintenance of Hematopoietic Stem Cells in Adult Bone Marrow. *Stem Cells and Development*, *24*(16), 1865-1877. https://doi.org/10.1089/scd.2015.0058

Förster, M., Boora, R. K., Petrov, J. C., Fodil, N., Albanese, I., Kim, J., Gros, P., & Nijnik, A. (2017). A role for the histone H2A deubiquitinase MYSM1 in maintenance of CD8+ T cells. *Immunology*, *151*(1), 110-121. https://doi.org/10.1111/imm.12710

Förster, M., Farrington, K., Petrov, J. C., Belle, J. I., Mindt, B. C., Witalis, M., Duerr, C. U., Fritz, J. H., & Nijnik, A. (2017). MYSM1-dependent checkpoints in B cell lineage differentiation and B cell–mediated immune response. *Journal of Leukocyte Biology*, *101*(3), 643-654. https://doi.org/10.1189/jlb.1AB0415-177RR

Fradet-Turcotte, A., Canny, M. D., Escribano-Díaz, C., Orthwein, A., Leung, C. C. Y., Huang, H., Landry, M.-C., Kitevski-Leblanc, J., Noordermeer, S. M., Sicheri, F., & Durocher, D. (2013). 53BP1 is a reader of the DNA-damage-induced H2A Lys 15 ubiquitin mark. *Nature*, *499*(7456), 50-54. https://doi.org/10.1038/nature12318

Fugmann, S. D., Lee, A. I., Shockett, P. E., Villey, I. J., & Schatz, D. G. (2000). The RAG proteins and V(D)J recombination: complexes, ends, and transposition. *Annu Rev Immunol*, *18*, 495-527. https://doi.org/10.1146/annurev.immunol.18.1.495

Gatti, M., Pinato, S., Maiolica, A., Rocchio, F., Prato, M. G., Aebersold, R., & Penengo, L. (2015). RNF168 promotes noncanonical K27ubiquitination to signal DNA damage. *Cell Reports*, *10*(2), 226-238. https://doi.org/10.1016/j.celrep.2014.12.021

Ginjala, V., Nacerddine, K., Kulkarni, A., Oza, J., Hill, S. J., Yao, M., Citterio, E., van Lohuizen, M., & Ganesan, S. (2011). BMI1 Is Recruited to DNA Breaks and Contributes to DNA Damage-Induced H2A Ubiquitination and Repair ▿ . *Molecular and Cellular Biology*, *31*(10), 1972-1982. https://doi.org/10.1128/MCB.00981-10

Glynn, R. A., & Bassing, C. H. (2022). Nemo-Dependent, ATM-Mediated Signals from RAG DNA Breaks at Igk Feedback Inhibit V κ Recombination to Enforce Igκ Allelic Exclusion. *The Journal of Immunology*, *208*(2), 371-383. https://doi.org/10.4049/jimmunol.2100696

Gyory, I., Boller, S., Nechanitzky, R., Mandel, E., Pott, S., Liu, E., & Grosschedl, R. (2012). Transcription factor Ebf1 regulates differentiation stage-specific signaling, proliferation, and survival of B cells. *Genes Dev*, *26*(7), 668-682. https://doi.org/10.1101/gad.187328.112

Helmink, B. A., & Sleckman, B. P. (2012). The Response to and Repair of RAG-Mediated DNA Double-Strand Breaks. *Annual Review of Immunology*, *30*(1), 175-202. https://doi.org/10.1146/annurev-immunol-030409-101320

Huang, K. Y., Amodeo, G. A., Tong, L., & Mcdermott, A. (2011). The structure of human ubiquitin in 2-methyl-2,4-pentanediol: A new conformational switch. *Protein Science*, *20*(3), 630-639. https://doi.org/10.1002/pro.584

Huen, M. S. Y., Grant, R., Manke, I., Minn, K., Yu, X., Yaffe, M. B., & Chen, J. (2007). RNF8 Transduces the DNA-Damage Signal via Histone Ubiquitylation and Checkpoint Protein Assembly. *Cell*, *131*(5), 901-914. https://doi.org/10.1016/j.cell.2007.09.041

Huo, Y., Li, B.-y., Lin, Z.-f., Wang, W., Jiang, X.-X., Chen, X., Xi, W.-J., Li, Y.-F., Yang, A.-g., Chen, S.-y., & Wang, T. (2018). MYSM1 Is Essential for Maintaining Hematopoietic Stem Cell (HSC) Quiescence and Survival. *Medical Science Monitor*, *24*, 2541-2549. https://doi.org/10.12659/MSM.906876

Ikeda, F. (2023). Protein and non-protein targets of ubiquitin modification. *American Journal of Physiology-Cell Physiology*, *252*.

Innes, C. L., Hesse, J. E., Morales, A. J., Helmink, B. A., Schurman, S. H., Sleckman, B. P., & Paules, R. S. (2020). DNA damage responses in murine Pre-B cells with genetic deficiencies in damage response genes. *Cell Cycle*, *19*(1), 67-83. https://doi.org/10.1080/15384101.2019.1693118

Ismail, I. H., Andrin, C., McDonald, D., & Hendzel, M. J. (2010). BMI1-mediated histone ubiquitylation promotes DNA double-strand break repair. *Journal of Cell Biology*, *191*(1), 45-60. https://doi.org/10.1083/jcb.201003034

Jiang, X. X., Nguyen, Q., Chou, Y. C., Wang, T., Nandakumar, V., Yates, P., Jones, L., Wang, L., Won, H., Lee, H. R., Jung, J. U., Müschen, M., Huang, X. F., & Chen, S. Y. (2011). Control of B Cell Development by the Histone H2A Deubiquitinase MYSM1. *Immunity*, *35*(6), 883-896. https://doi.org/10.1016/j.immuni.2011.11.010

Jin, Z., Zheng, E., Sareli, C., Kolattukudy, P. E., & Niu, J. (2021). Monocyte Chemotactic Protein-Induced Protein 1 (MCPIP-1): A Key Player of Host Defense and Immune Regulation. *Front Immunol*, *12*, 727861. https://doi.org/10.3389/fimmu.2021.727861

Johnston, R., Mathias, B., Crowley, S. J., Schmidt, H. A., White, L. S., Mosammaparast, N., Green, A. M., & Bednarski, J. J. (2023). Nuclease-independent functions of RAG1 direct distinct DNA damage responses in B cells. *EMBO reports*, *24*(1), e55429. https://doi.org/10.15252/embr.202255429

Johnston, R., White, L. S., & Bednarski, J. J. (2022). Assessing DNA Damage Responses Using B Lymphocyte Cultures. In *Methods in Molecular Biology* (pp. 69-80). Springer US. https://doi.org/10.1007/978-1-0716-2063-2_5

Kadimisetty, K., Sheets, K. J., Gross, P. H., Zerr, M. J., & Ouazia, D. (2021). Tandem Ubiquitin Binding Entities (TUBEs) as Tools to Explore Ubiquitin-Proteasome System and PROTAC Drug Discovery. In *Targeted Protein Degradation* (pp. 185-202). Springer US. https://doi.org/10.1007/978-1-0716-1665-9_10

Kaiho-Soma, A., Akizuki, Y., Igarashi, K., Endo, A., Shoda, T., Kawase, Y., Demizu, Y., Naito, M., Saeki, Y., Tanaka, K., & Ohtake, F. (2021). TRIP12 promotes small-molecule-induced degradation through K29/K48-branched ubiquitin chains. *Molecular Cell*, *81*(7), 1411-1424.e1417. https://doi.org/10.1016/J.MOLCEL.2021.01.023

Kakarougkas, A., Ismail, A., Katsuki, Y., Freire, R., Shibata, A., & Jeggo, P. A. (2013). Co-operation of BRCA1 and POH1 relieves the barriers posed by 53BP1 and RAP80 to resection. *Nucleic Acids Research*, *41*(22), 10298-10311. https://doi.org/10.1093/nar/gkt802

Kim, M. S., Lapkouski, M., Yang, W., & Gellert, M. (2015). Crystal structure of the V(D)J recombinase RAG1-RAG2. *Nature*, *518*(7540), 507-511. https://doi.org/10.1038/nature14174

Kim, S.-T., Lim, D.-S., Canman, C. E., & Kastan, M. B. (1999). Substrate Specificities and Identification of Putative Substrates of ATM Kinase Family Members. *Journal of Biological Chemistry*, *274*(53), 37538-37543. https://doi.org/10.1074/jbc.274.53.37538

Kitagawa, R., & Kastan, M. B. (2005). The ATM-dependent DNA damage signaling pathway. *Cold Spring Harbor Symposia on Quantitative Biology*, *70*, 99-109. https://doi.org/10.1101/sqb.2005.70.002

Kollarovic, G., Topping, C. E., Shaw, E. P., & Chambers, A. L. (2020). The human HELLS chromatin remodelling protein promotes end resection to facilitate homologous recombination and contributes to DSB repair within heterochromatin. *Nucleic Acids Res*, *48*(4), 1872-1885. https://doi.org/10.1093/nar/gkz1146

Kroeger, C., Roessler, R., Wiese, S., Hainzl, A., & Gatzka, M. V. (2020). Interaction of deubiquitinase 2A-DUB/MYSM1 with DNA repair and replication factors. *International Journal of Molecular Sciences*, *21*(11). https://doi.org/10.3390/ijms21113762

Lancini, C., van den Berk, P. C., Vissers, J. H., Gargiulo, G., Song, J. Y., Hulsman, D., Serresi, M., Tanger, E., Blom, M., Vens, C., van Lohuizen, M., Jacobs, H., & Citterio, E. (2014). Tight regulation of ubiquitin-mediated DNA damage response by USP3 preserves the functional integrity of hematopoietic stem cells. *J Exp Med*, *211*(9), 1759-1777. https://doi.org/10.1084/jem.20131436

Lauinger, L., Li, J., Shostak, A., Cemel, I. A., Ha, N., Zhang, Y., Merkl, P. E., Obermeyer, S., Stankovic-Valentin, N., Schafmeier, T., Wever, W. J., Bowers, A. A., Carter, K. P., Palmer, A. E., Tschochner, H., Melchior, F., Deshaies, R. J., Brunner, M., & Diernfellner, A. (2017). Thiolutin is a zinc chelator that inhibits the Rpn11 and other JAMM metalloproteases. *Nature Chemical Biology*, *13*(7), 709-714. https://doi.org/10.1038/nchembio.2370

Le Guen, T., Touzot, F., André-Schmutz, I., Lagresle-Peyrou, C., France, B., Kermasson, L., Lambert, N., Picard, C., Nitschke, P., Carpentier, W., Bole-Feysot, C., Lim, A., Cavazzana, M., Callebaut, I., Soulier, J., Jabado, N., Fischer, A., De Villartay, J. P., & Revy, P. (2015). An in vivo genetic reversion highlights the crucial role of Myb-Like, SWIRM, and MPN domains 1 (MYSM1) in human hematopoiesis and lymphocyte differentiation. *Journal of Allergy and Clinical Immunology*, *136*(6), 1619-1626e1615. https://doi.org/10.1016/j.jaci.2015.06.008

Lee, J.-H., Goodarzi, A. A., Jeggo, P. A., & Paull, T. T. (2010). 53BP1 promotes ATM activity through direct interactions with the MRN complex. *The EMBO Journal*, *29*(3), 574-585. https://doi.org/10.1038/emboj.2009.372

Lenden Hasse, H., Lescale, C., Bianchi, J. J., Yu, W., Bedora-Faure, M., & Deriano, L. (2017). Generation and CRISPR/Cas9 editing of transformed progenitor B cells as a pseudo-physiological system to study DNA repair gene function in V(D)J recombination. *Journal of Immunological Methods*, *451*, 71-77. https://doi.org/10.1016/j.jim.2017.08.007

Li, N., Xu, Y., Yu, T., Yao, R., Chen, J., Luo, C., & Wang, J. (2020). Further delineation of bone marrow failure syndrome caused by novel compound heterozygous variants of MYSM1. *Gene*, *757*, 144938. https://doi.org/10.1016/j.gene.2020.144938

Liang, J., Saad, Y., Lei, T., Wang, J., Qi, D., Yang, Q., Kolattukudy, P. E., & Fu, M. (2010). MCP-induced protein 1 deubiquitinates TRAF proteins and negatively regulates JNK and NF-κB signaling. *Journal of Experimental Medicine*, *207*(13), 2959-2973. https://doi.org/10.1084/jem.20092641

Liang, Y., Bhatt, G., Tung, L. T., Wang, H. C., Kim, J. E., Mousa, M., Plackoska, V., Illes, K., Georges, A. A., Gros, P., Henneman, L., Huijbers, I. J., Nagar, B., & Nijnik, A. (2023). Deubiquitinase catalytic activity of MYSM1 is essential in vivo for hematopoiesis and immune cell development. *Scientific Reports*, *13*(1). https://doi.org/10.1038/s41598-023-27486-7

Lu, Z., Hong, C. C., Kong, G., Assumpção, A. L. F. V., Ong, I. M., Bresnick, E. H., Zhang, J., & Pan, X. (2018). Polycomb Group Protein YY1 Is an Essential Regulator of Hematopoietic Stem Cell Quiescence. *Cell Reports*, *22*(6), 1545-1559. https://doi.org/10.1016/j.celrep.2018.01.026

Ma, Y., Pannicke, U., Schwarz, K., & Lieber, M. R. (2002). Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. *Cell*, *108*(6), 781-794. https://doi.org/10.1016/S0092-8674(02)00671-2

Mailand, N., Bekker-Jensen, S., Faustrup, H., Melander, F., Bartek, J., Lukas, C., & Lukas, J. (2007). RNF8 Ubiquitylates Histones at DNA Double-Strand Breaks and Promotes Assembly of Repair Proteins. *Cell*, *131*(5), 887-900. https://doi.org/10.1016/j.cell.2007.09.040

Makukhin, N., & Ciulli, A. (2021). Recent advances in synthetic and medicinal chemistry of phosphotyrosine and phosphonate-based phosphotyrosine analogues. *RSC Medicinal Chemistry*, *12*(1), 8-23. https://doi.org/10.1039/d0md00272k

Mandel, E. M., & Grosschedl, R. (2010). Transcription control of early B cell differentiation. *Current Opinion in Immunology*, *22*(2), 161-167. https://doi.org/10.1016/j.coi.2010.01.010

Maréchal, A., & Zou, L. (2015). RPA-coated single-stranded DNA as a platform for post-translational modifications in the DNA damage response. *Cell Research*, *25*(1), 9-23. https://doi.org/10.1038/cr.2014.147

Matsui, M., Kajita, S., Tsuchiya, Y., Torii, W., Tamekuni, S., & Nishi, R. (2022). USP49 is a novel deubiquitylating enzyme for γ H2AX in DNA double-strand break repair. *Gene*, *833*. https://doi.org/10.1016/j.gene.2022.146599

Matsuoka, S., Ballif, B. A., Smogorzewska, A., Mcdonald Iii, E. R., Hurov, K. E., Luo, J., Bakalarski, C. E., Zhao, Z., Solimini, N., Lerenthal, Y., Shiloh, Y., Gygi, S. P., & Elledge, S. J. (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science*, *316*(May), 1160-1167. https://doi.org/10.1126/science.1140321

Matsuoka, S., Rotman, G., Ogawa, A., Shiloh, Y., Tamai, K., & Elledge, S. J. (2000). Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(19), 10389-10394. https://doi.org/10.1073/pnas.190030497

Mattiroli, F., Vissers, J. H. A., Van Dijk, W. J., Ikpa, P., Citterio, E., Vermeulen, W., Marteijn, J. A., & Sixma, T. K. (2012). RNF168 ubiquitinates K13-15 on H2A/H2AX to drive DNA damage signaling. *Cell*, *150*(6), 1182-1195. https://doi.org/10.1016/j.cell.2012.08.005

Mayya, V., Lundgren, D. H., Hwang, S.-I., Rezaul, K., Wu, L., Eng, J. K., Rodionov, V., & Han, D. K. (2009). Quantitative Phosphoproteomic Analysis of T Cell Receptor Signaling Reveals System-Wide Modulation of Protein-Protein Interactions. *Science Signaling*, *2*(84). https://doi.org/10.1126/scisignal.2000007

Meek, D. W. (2004). The p53 response to DNA damage. *DNA Repair (Amst)*, *3*(8-9), 1049-1056. https://doi.org/10.1016/j.dnarep.2004.03.027

Melchionna, R., Chen, X.-B., Blasina, A., & McGowan, C. H. (2000). Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. *Nature Cell Biology*, *2*(10), 762-765. https://doi.org/10.1038/35036406

Mennerich, D., Kubaichuk, K., & Kietzmann, T. (2019). DUBs, Hypoxia, and Cancer. *Trends in Cancer*, *5*(10), 632-653. https://doi.org/10.1016/j.trecan.2019.08.005

Metzger, M. B., Hristova, V. A., & Weissman, A. M. (2012). HECT and RING finger families of E3 ubiquitin ligases at a glance. *Journal of Cell Science*, *125*(3), 531-537. https://doi.org/10.1242/jcs.091777

Miyamoto, S. (2011). Nuclear initiated NF-κB signaling: NEMO and ATM take center stage. *Cell Research*, *21*(1), 116-130. https://doi.org/10.1038/cr.2010.179

Mosbech, A., Lukas, C., Bekker-Jensen, S., & Mailand, N. (2013). The deubiquitylating enzyme USP44 counteracts the DNA double-strand break response mediated by the RNF8 and RNF168 ubiquitin ligases. *J Biol Chem*, *288*(23), 16579-16587. https://doi.org/10.1074/jbc.M113.459917

Moshous, D., Callebaut, I., Chasseval, D., Corneo, B., Cavazzana-calvo, M., Deist, L., Tezcan, I., Sanal, O., Bertrand, Y., Philippe, N., Fischer, A., & Villartay, J.-p. D. (2001). Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. *Cell*, *105*(D), 177-186.

Murphy, K., & Weaver, C. (2017). *Immunobiology*.

Ng, H. M., Wei, L., Lan, L., & Huen, M. S. Y. (2016). The Lys63-deubiquitylating enzyme BRCC36 limits DNA break processing and repair. *Journal of Biological Chemistry*, *291*(31), 16197-16207. https://doi.org/10.1074/jbc.M116.731927

Nijnik, A., Simon Clare, C. H., Raisen, C., McIntyre, R. E., Yusa, K., Everitt, A. R., Mottram, L., Podrini, C., Lucas, M., Estabel, J., Goulding, D., Adams, N., Ramirez-Solis, R., White, J. K., Adams, D. J., Hancock, R. E. W., & Dougan, G. (2012). The critical role of histone H2A-deubiquitinase Mysm1 in hematopoiesis and lymphocyte differentiation. *Blood*, *119*(6), 1370-1379. https://doi.org/10.1182/blood-2011-05-352666

Nishi, R., Wijnhoven, P., Le Sage, C., Tjeertes, J., Galanty, Y., Forment, J. V., Clague, M. J., Urbé, S., & Jackson, S. P. (2014). Systematic characterization of deubiquitylating enzymes for roles in maintaining genome integrity. *Nature Cell Biology*, *16*(10), 1016-1026. https://doi.org/10.1038/ncb3028

O'Neill, T., Dwyer, A. J., Ziv, Y., Chan, D. W., Lees-Miller, S. P., Abraham, R. H., Lai, J. H., Hill, D., Shiloh, Y., Cantley, L. C., & Rathbun, G. A. (2000). Utilization of Oriented Peptide Libraries to Identify Substrate Motifs Selected by ATM. *Journal of Biological Chemistry*, *275*(30), 22719-22727. https://doi.org/10.1074/jbc.m001002200

Ochoa, D., Jarnuczak, A. F., Viéitez, C., Gehre, M., Soucheray, M., Mateus, A., Kleefeldt, A. A., Hill, A., Garcia-Alonso, L., Stein, F., Krogan, N. J., Savitski, M. M., Swaney, D. L., Vizcaíno, J. A., Noh, K.-M., & Beltrao, P. (2020). The functional landscape of the human phosphoproteome. *Nature Biotechnology*, *38*(3), 365-373. https://doi.org/10.1038/s41587-019-0344-3

Ohtake, F., Saeki, Y., Ishido, S., Kanno, J., & Tanaka, K. (2016). The K48-K63 Branched Ubiquitin Chain Regulates NF-κB Signaling. *Molecular Cell*, *64*(2), 251-266. https://doi.org/10.1016/J.MOLCEL.2016.09.014

Olsson, A., Manzl, C., Strasser, A., & Villunger, A. (2007). How important are post-translational modifications in p53 for selectivity in target-gene transcription and tumour suppression? *Cell Death & Differentiation*, *14*(9), 1561-1575. https://doi.org/10.1038/sj.cdd.4402196

Paiano, J., Zolnerowich, N., Wu, W., Pavani, R., Wang, C., Li, H., Zheng, L., Shen, B., Sleckman, B. P., Chen, B.-R., & Nussenzweig, A. (2021). Role of 53BP1 in end protection and DNA synthesis at DNA breaks. *Genes & Development*, *35*(19-20), 1356-1367. https://doi.org/10.1101/gad.348667.121

Pan, M.-R., Peng, G., Hung, W.-C., & Lin, S.-Y. (2011). Monoubiquitination of H2AX Protein Regulates DNA Damage Response Signaling. *Journal of Biological Chemistry*, *286*(32), 28599-28607. https://doi.org/10.1074/jbc.m111.256297

Pan, X., Wu, S., Wei, W., Chen, Z., Wu, Y., & Gong, K. (2022). Structural and Functional Basis of JAMM Deubiquitinating Enzymes in Disease. *Biomolecules*, *12*(7), 910. https://doi.org/10.3390/biom12070910

Panda, S., & Gekara, N. O. (2018). The deubiquitinase MYSM1 dampens NOD2-mediated inflammation and tissue damage by inactivating the RIP2 complex. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-07016-0

Panda, S., Nilsson, J. A., & Gekara, N. O. (2015). Deubiquitinase MYSM1 Regulates Innate Immunity through Inactivation of TRAF3 and TRAF6 Complexes. *Immunity*, *43*(4), 647-659. https://doi.org/10.1016/j.immuni.2015.09.010

Pickart, C. M., & Eddins, M. J. (2004). Ubiquitin: Structures, functions, mechanisms. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1695*(1-3), 55-72. https://doi.org/10.1016/j.bbamcr.2004.09.019

Plevin, M. J., Mills, M. M., & Ikura, M. (2005). The LxxLL motif: a multifunctional binding sequence in transcriptional regulation. *Trends in Biochemical Sciences, 30*(2), 66-69. https://doi.org/10.1016/j.tibs.2004.12.001

Rass, E., Willaume, S., & Bertrand, P. (2022). 53BP1: Keeping It under Control, Even at a Distance from DNA Damage. *Genes, 13*(12), 2390. https://doi.org/10.3390/genes13122390

Riley, B. E., Lougheed, J. C., Callaway, K., Velasquez, M., Brecht, E., Nguyen, L., Shaler, T., Walker, D., Yang, Y., Regnstrom, K., Diep, L., Zhang, Z., Chiou, S., Bova, M., Artis, D. R., Yao, N., Baker, J., Yednock, T., & Johnston, J. A. (2013). Structure and function of Parkin E3 ubiquitin ligase reveals aspects of RING and HECT ligases. *Nature Communications*, *4*(1). https://doi.org/10.1038/ncomms2982

Rizo, A., Olthof, S., Han, L., Vellenga, E., de Haan, G., & Schuringa, J. J. (2009). Repression of BMI1 in normal and leukemic human CD34(+) cells impairs self-renewal and induces apoptosis. *Blood*, *114*(8), 1498-1505. https://doi.org/10.1182/blood-2009-03-209734

Rizzotto, D., Englmaier, L., & Villunger, A. (2021). At a Crossroads to Cancer: How p53-Induced Cell Fate Decisions Secure Genome Integrity. *International Journal of Molecular Sciences*, *22*(19), 10883. https://doi.org/10.3390/ijms221910883

Sato, Y., Yoshikawa, A., Yamagata, A., Mimura, H., Yamashita, M., Ookata, K., Nureki, O., Iwai, K., Komada, M., & Fukai, S. (2008). Structural basis for specific cleavage of Lys 63-linked polyubiquitin chains. *Nature*, *455*(7211), 358-362. https://doi.org/10.1038/nature07254

Savkur, R. S., & Burris, T. P. (2004). The coactivator LXXLL nuclear receptor recognition motif. *J Pept Res*, *63*(3), 207-212. https://doi.org/10.1111/j.1399-3011.2004.00126.x

Sharma, A., & Almasan, A. (2020). USP14 regulates dna damage response and is a target for radiosensitization in non-small cell lung cancer. *International Journal of Molecular Sciences*, *21*(17), 1-15. https://doi.org/10.3390/ijms21176383

Sharma, A., Alswillah, T., Singh, K., Chatterjee, P., Willard, B., Venere, M., Summers, M. K., & Almasan, A. (2018). USP14 regulates DNA damage repair by targeting RNF168-dependent ubiquitination. *Autophagy*, *14*(11), 1976-1990. https://doi.org/10.1080/15548627.2018.1496877

Shibata, A., Jeggo, P., & Lobrich, M. (2018). The pendulum of the Ku-Ku clock. *DNA Repair (Amst)*, *71*, 164-171. https://doi.org/10.1016/j.dnarep.2018.08.020

Shibata, A., & Jeggo, P. A. (2020a). Canonical DNA non-homologous end-joining; capacity versus fidelity. *Br J Radiol*, *93*(1115), 20190966. https://doi.org/10.1259/bjr.20190966

Shibata, A., & Jeggo, P. A. (2020b). Roles for 53BP1 in the repair of radiation-induced DNA double strand breaks. *DNA Repair (Amst)*, *93*, 102915. https://doi.org/10.1016/j.dnarep.2020.102915

Shiloh, Y., & Ziv, Y. (2013). The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nat Rev Mol Cell Biol*, *14*(4), 197-210. https://doi.org/10.1038/nrm3546

Simpson, E., & Dazzi, F. (2019). Bone Marrow Transplantation 1957-2019. *Frontiers in Immunology*, *10*. https://doi.org/https://doi.org/10.3389/fimmu.2019.01246

Snyder, N. A., & Silva, G. M. (2021). Deubiquitinating enzymes (DUBs): Regulation, homeostasis, and oxidative stress response. *Journal of Biological Chemistry*, *297*(3), 101077. https://doi.org/10.1016/j.jbc.2021.101077

Soodgupta, D., White, L. S., Yang, W., Johnston, R., Mosammaparast, N., Payton, J. E., & Bednarski, J. (2019). RAG-Mediated DNA Breaks Attenuate PU.1 Activity in Early B Cells Through Activation of a SPIC-BCLAF1 Complex. *SSRN Electronic Journal*. https://doi.org/10.2139/ssrn.3362390

Stewart, S. A., Dykxhoorn, D. M., Palliser, D., Mizuno, H., Yu, E. Y., An, D. S., Sabatini, D. M., Chen, I. S. Y., Hahn, W. C., Sharp, P. A., Weinberg, R. A., & Novina, C. D. (2003). Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA*, *9*(4), 493-501. https://doi.org/10.1261/rna.2192803

Swatek, K. N., & Komander, D. (2016). Ubiquitin modifications. *Cell Research*, *26*(4), 399-422. https://doi.org/10.1038/cr.2016.39

Syed, A., & Tainer, J. A. (2018). The MRE11-RAD50-NBS1 Complex Conducts the Orchestration of Damage Signaling and Outcomes to Stress in DNA Replication and Repair. *Annu Rev Biochem*, *87*, 263-294. https://doi.org/10.1146/annurev-biochem-062917-012415

Tarsounas, M., & Sung, P. (2020). The antitumorigenic roles of BRCA1-BARD1 in DNA repair and replication. *Nat Rev Mol Cell Biol*, *21*(5), 284-299. https://doi.org/10.1038/s41580-020-0218-z

Teng, G., & Schatz, D. G. (2015). Regulation and Evolution of the RAG Recombinase. In *Advances in Immunology* (Vol. 128, pp. 1-39). Academic Press Inc.

Tian, M., Liu, W., Zhang, Q., Huang, Y., Li, W., Wang, W., Zhao, P., Huang, S., Song, Y., Shereen, M. A., Qin, M., Liu, Y., Wu, K., & Wu, J. (2020). MYSM1 Represses Innate Immunity and Autoimmunity through Suppressing the cGAS-STING Pathway. *Cell Reports*, *33*(3), 108297. https://doi.org/10.1016/j.celrep.2020.108297

Ting, X., Xia, L., Yang, J., He, L., Si, W., Shang, Y., & Sun, L. (2019). USP11 acts as a histone deubiquitinase functioning in chromatin reorganization during DNA repair. *Nucleic Acids Research*, *47*(18), 9721-9740. https://doi.org/10.1093/nar/gkz726

Typas, D., Luijsterburg, M. S., Wiegant, W. W., Diakatou, M., Helfricht, A., Thijssen, P. E., van den Broek, B., Mullenders, L. H., & van Attikum, H. (2015). The de-ubiquitylating enzymes USP26 and USP37 regulate homologous recombination by counteracting RAP80. *Nucleic Acids Res*, *43*(14), 6919-6933. https://doi.org/10.1093/nar/gkv613

Uckelmann, M., & Sixma, T. K. (2017). Histone ubiquitination in the DNA damage response. *DNA Repair*, *56*(June), 92-101. https://doi.org/10.1016/j.dnarep.2017.06.011

Vidal, M., & Starowicz, K. (2017). Polycomb complexes PRC1 and their function in hematopoiesis. *Experimental Hematology*, *48*, 12-31. https://doi.org/10.1016/j.exphem.2016.12.006

Vilagos, B., Hoffmann, M., Souabni, A., Sun, Q., Werner, B., Medvedovic, J., Bilic, I., Minnich, M., Axelsson, E., Jaritz, M., & Busslinger, M. (2012). Essential role of EBF1 in the generation and function of distinct mature B cell types. *The Journal of Experimental Medicine*, *209*(4), 775-792. https://doi.org/10.1084/jem.20112422

Vissers, J. H. A., Nicassio, F., van Lohuizen, M., Di Fiore, P. P., & Citterio, E. (2008). The many faces of ubiquitinated histone H2A: Insights from the DUBs. *Cell Division*, *3*, 1-14. https://doi.org/10.1186/1747-1028-3-8

Walser, F., Mulder, M. P. C., Bragantini, B., Burger, S., Gubser, T., Gatti, M., Botuyan, M. V., Villa, A., Altmeyer, M., Neri, D., Ovaa, H., Mer, G., & Penengo, L. (2020). Ubiquitin Phosphorylation at Thr12 Modulates the DNA Damage Response. *Molecular Cell*, *80*(3), 423-436.e429. https://doi.org/10.1016/j.molcel.2020.09.017

Wang, B., & Elledge, S. J. (2007). Ubc13/Rnf8 ubiquitin ligases control foci formation of the Rap80/Abraxas/Brca1/Brcc36 complex in response to DNA damage. *Proceedings of the National Academy of Sciences*, *104*(52), 20759-20763. https://doi.org/10.1073/pnas.0710061104

Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R. S., & Zhang, Y. (2004). Role of histone H2A ubiquitination in Polycomb silencing. *Nature*, *431*(7010), 873-878. https://doi.org/10.1038/nature02985

Wang, T., Nandakumar, V., Jiang, X.-X., Jones, L., Yang, A.-G., Huang, X. F., & Chen, S.-Y. (2013). The control of hematopoietic stem cell maintenance, self-renewal, and differentiation by Mysm1-mediated epigenetic regulation. *Blood*, *122*(16), 2812-2822. https://doi.org/10.1182/blood-2013-03-489641

Wang, X., Mazurkiewicz, M., Hillert, E. K., Olofsson, M. H., Pierrou, S., Hillertz, P., Gullbo, J., Selvaraju, K., Paulus, A., Akhtar, S., Bossler, F., Khan, A. C., Linder, S., & D'Arcy, P. (2016). The proteasome deubiquitinase inhibitor VLX1570 shows selectivity for ubiquitin-specific protease-14 and induces apoptosis of multiple myeloma cells. *Scientific Reports*, *6*(November 2015), 1-15. https://doi.org/10.1038/srep26979

Wang, Z., Zhang, H., Liu, J., Cheruiyot, A., Lee, J.-H., Ordog, T., Lou, Z., You, Z., & Zhang, Z. (2016). USP51 deubiquitylates H2AK13,15ub and regulates DNA damage response. *Genes & Development*, *30*(8), 946-959. https://doi.org/10.1101/gad.271841.115

Wheaton, K., Sarkari, F., Johns, B. S., Davarinejad, H., Egorova, O., Kaustov, L., Raught, B., Saridakis, V., & Sheng, Y. (2017). UbE2E1/UBCH6 Is a Critical in Vivo E2 for the PRC1-catalyzed Ubiquitination of H2A at Lys-119. *Journal of Biological Chemistry*, *292*(7), 2893-2902. https://doi.org/10.1074/jbc.M116.749564

White, D. E., Negorev, D., Peng, H., Ivanov, A. V., Maul, G. G., & Rauscher, F. J., III. (2006). KAP1, a Novel Substrate for PIKK Family Members, Colocalizes with Numerous Damage Response Factors at DNA Lesions. *Cancer Research*, *66*(24), 11594-11599. https://doi.org/10.1158/0008-5472.CAN-06-4138

Wilson, M. D., Benlekbir, S., Fradet-Turcotte, A., Sherker, A., Julien, J. P., McEwan, A., Noordermeer, S. M., Sicheri, F., Rubinstein, J. L., & Durocher, D. (2016). The structural basis of modified nucleosome recognition by 53BP1. *Nature*, *536*(7614), 100-103. https://doi.org/10.1038/nature18951

Yoneyama, M., Tochio, N., Umehara, T., Koshiba, S., Inoue, M., Yabuki, T., Aoki, M., Seki, E., Matsuda, T., Watanabe, S., Tomo, Y., Nishimura, Y., Harada, T., Terada, T., Shirouzu, M., Hayashizaki, Y., Ohara, O., Tanaka, A., Kigawa, T., & Yokoyama, S. (2007). Structural and Functional Differences of SWIRM Domain Subtypes. *Journal of Molecular Biology*, *369*(1), 222-238. https://doi.org/10.1016/j.jmb.2007.03.027

Yu, M., Mazor, T., Huang, H., Huang, H.-T., Kathrein, L., Katie, Woo, J., Andrew, Chouinard, R., Candace, Labadorf, A., Akie, E., Thomas, Moran, B., Tyler, Xie, H., Zacharek, S., Taniuchi, I., Roeder, G., Robert, Kim, F., Carla, Zon, I., Leonard, Fraenkel, E., & Cantor, B., Alan. (2012). Direct Recruitment of Polycomb Repressive Complex 1 to Chromatin by Core Binding Transcription Factors. *Molecular Cell*, *45*(3), 330-343. https://doi.org/10.1016/j.molcel.2011.11.032

Zandi, S., Mansson, R., Tsapogas, P., Zetterblad, J., Bryder, D., & Sigvardsson, M. (2008). EBF1 Is Essential for B-Lineage Priming and Establishment of a Transcription Factor Network in Common Lymphoid Progenitors. *The Journal of Immunology*, *181*(5), 3364-3372. https://doi.org/10.4049/jimmunol.181.5.3364

Zhao, Y., Mudge, M. C., Soll, J. M., Rodrigues, R. B., Byrum, A. K., Schwarzkopf, E. A., Bradstreet, T. R., Gygi, S. P., Edelson, B. T., & Mosammaparast, N. (2018). OTUD4 Is a Phospho-Activated K63 Deubiquitinase that Regulates MyD88-Dependent Signaling. *Molecular Cell*, *69*(3), 505-515.e505. https://doi.org/10.1016/j.molcel.2018.01.009

Zheng, N., & Shabek, N. (2017). Ubiquitin Ligases: Structure, Function, and Regulation. *Annual Review of Biochemistry*, *86*(1), 129-157. https://doi.org/10.1146/annurev-biochem-060815-014922

Zhou, H., Di Palma, S., Preisinger, C., Peng, M., Polat, A. N., Heck, A. J. R., & Mohammed, S. (2013). Toward a Comprehensive Characterization of a Human Cancer Cell Phosphoproteome. *Journal of Proteome Research*, *12*(1), 260-271. https://doi.org/10.1021/pr300630k

Zhu, P., Zhou, W., Wang, J., Puc, J., Ohgi, K. A., Erdjument-Bromage, H., Tempst, P., Glass, C. K., & Rosenfeld, M. G. (2007). A Histone H2A Deubiquitinase Complex Coordinating Histone Acetylation and H1 Dissociation in Transcriptional Regulation. *Molecular Cell*, *27*(4), 609-621. https://doi.org/10.1016/j.molcel.2007.07.024

Ziv, Y., Bielopolski, D., Galanty, Y., Lukas, C., Taya, Y., Schultz, D. C., Lukas, J., Bekker-Jensen, S., Bartek, J., & Shiloh, Y. (2006). Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. *Nature Cell Biology*, *8*(8), 870-876. https://doi.org/10.1038/ncb1446