Understanding the regulation of p53 transcriptional activity via MYSM1

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List of abbreviations

Cdk2	Cell division stimulating protein 2
CHD3 and 4	Chromodomain-helicase-DNA-binding protein 3 and 4
ChIP	Chromatin immunoprecipitation
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DUB	De-ubiquitinase
Ebf1	Early B-cell factor 1
GATAD2A and B	GATA Binding Protein 2
Gfi1	Growth factor independent 1
HAT	Histone acetyltransferase
HDAC1	Histone deacetylase 1
HDAC2	Histone deacetylase 2
HSC	Hematopoietic stem cell
H2Aub	Histone H2A ubiquitination
IBMFS	Inherited bone marrow failure syndromes
Immunoglobulin	Ig
JAMM domain	JAB1/MPN/Mov34 metalloenzyme domain
K(#)	Lysine(amino acid position)
K119Ub	Lysine 119 ubiquitination
K4me	Lysine 4 methylation
K4me3	Lysine 27 tri-methylation
K9me3	Lysine 9 tri-methylation
K27ac	Lysine 27 acetylation
K27me3	Lysine 27 tri-methylation
KLS	Kit+Lineage-Sca1+
MBD2 and 3	Methyl-CpG-binding domain
MDM2	Mouse double minute-2
MDM4	Mouse double minute-4
MJDP	Machado-Joseph disease proteases
MPP	Multipotent progenitors
MTA1, 2, and 3	Metastasis-associated gene 1, 2, and 3
MYSM1	Myb-like swirm and MPN domains 1
NK	Natural killer
NuRD	Nucleosome remodeling and deacetylase complex
p53	Tumor protein 53
PARP	Poly ADP ribose polymerase

pCAF	P300/CBP-associated factor
PUMA	P53 upreglated modulator of apoptosis
RBBP4 and 7	Retinoblastoma-binding protein 4 and 7
qPCR	Quantitative polymerase chain reaction
Ser(#)	Serine(amino acid position)
UCH	Ubiquitin C-terminal hydrolases
USP	Ubiquitin-specific protease
OTU	Otubain proteases

Abstract

p53 is a tumor suppressor protein that is mutated in up to 50% of human cancers. It regulates the expression of hundreds of genes, which cause cell cycle arrest, cellular senescence, or apoptosis. We are hypothesizing that MYSM1, a chromatin interacting deubiquitinase, regulates the transcriptional activity of p53 by localizing to DNA with chromatin modifying factors, and controlling post-translational modifications of p53 or the chromatin at p53 target promoters. We recently demonstrated that loss of *Mysm1* results in p53 activation in the mouse hematopoietic system, while inactivation of p53 can fully rescue the phenotypic abnormalities seen in *Mysm1*-knockouts. Mass spectrometry data has shown that catalytic components of the NuRD complex, specifically HDAC1 and 2, interact with MYSM1. These components have also been shown to deacetylate p53. In this work, we performed protein-protein interaction studies in H1299 cells to confirm the binding of MYSM1 with different components of the NuRD complex. The effects of MYSM1 on p53 acetylation were then analyzed, using the *Mysm1*-knockdown Ba/F3 hematopoietic progenitor cell model, demonstrating that MYSM1 antagonizes p53-K379 acetylation. Following this, chromatin immunoprecipitation studies were done using Ba/F3 cells at steady state and after irradiation to test the effects of *Mysm1*-knockdown on the binding of HDAC1 and HDAC2 to the promoters of p53 target genes such as *Cdkn1a* and *Bbc3*. The results support the hypothesis that MYSM1 may promote HDAC2 recruitment to p53-binding sites at least within the *Bbc3* gene promoter. Overall the data supports our hypothesis that MYSM1 interacting with the NuRD complex may be mechanistically important for inhibition of p53 transcriptional activity in hematopoietic progenitors. This work is important in understanding the regulation of the p53 pathway in hematopoietic systems. Since p53 mutations are less common in hematological malignancies than in solid tumors, activation of p53 via MYSM1-inhibition may be a novel strategy for the treatment of hematological cancers.

Résumé

La protéine p53, mutée dans 50% des cancers humains, joue un rôle fondamental dans la suppression tumorale. Ce rôle est caractérisé par la modulation de plusieurs centaines de gènes cibles qui provoquent l'arrêt du cycle cellulaire, la sénescence et l'apoptose. Nous postulons que la protéine MYSM1, une désubiquitinase capable de se fixer à la chromatine, se localise à l'ADN en complexe avec d'autres facteurs de modification de la chromatine soit pour réguler l'activité transcriptionnelle et les modifications post-traductionnelles de p53, ou bien pour altérer la chromatine aux promoteurs cibles du gène *p53*. Chez la souris, nous avons récemment démontré que l'invalidation de MYSM1 déclenche l'activation de p53 dans le système hématopoïétique, tandis que les phénotypes anormaux observés dans les souris *knock-out* pour *Mysm1* disparaissent suite à l'inactivation de p53. En récoltant des données de spectrométrie de masse, nous avons constaté que MYSM1 interagit avec plusieurs protéines catalytiques membres du complexe NuRD, notamment HDAC1 et HDAC2. De plus, ces protéines sont capables de désacétyler p53. Afin de confirmer la liaison de MYSM1 avec différents constituants du complexe NuRD, des études d'interaction protéine-protéine ont été effectuées dans la lignée cellulaire H1299. En revanche, l'activité de MYSM1 au niveau de l'acétylation a été analysé dans les cellules Ba/F3. Cette lignée cellulaire a aussi permis l'étude des effets de MYSM1 sur la liaison de HDAC1 et HDAC2 aux promoteurs de gènes cibles de p53 (*Cdkn1a* et *Bbc3*), en employant l'immunoprécipitation de chromatine dans des cellules à l'état stable ou suite à l'irradiation. Dans l'ensemble, ce travail est important pour comprendre la régulation de p53 dans le système hématopoïétique. Étant donné que les mutations de p53 sont moins fréquentes dans les hémopathies malignes que dans les tumeurs solides, l'activation de p53 par l'inhibition de MYSM1 est une stratégie réalisable pour le traitement des cancers hématologiques.

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Acknowledgements

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In Brief

The Myb-Like, SWIRM and MPN Domains 1 (MYSM1) protein was originally discovered as a chromatin-binding deubiquitinase and has since been shown to be essential for hematopoiesis. Mice with a *Mysm1* deletion were shown to have hematopoietic defects as well as physical abnormalities such as lack of tail and reduced body size and weight. Interestingly, there was also an upregulation in the p53 protein levels in the early hematopoietic progenitors.

Mysm1$^{-/-}$*p53*$^{-/-}$ mice have a rescue of the *Mysm1*$^{-/-}$ hematopoietic and physical phenotype. We have shown that there is an interaction between MYSM1 and p53 proteins, as well as increased recruitment of p53 and activating histone acetylation marks at known p53-target gene promoters such as *Bbc3*/PUMA and *Cdkn1a*/p21 when MYSM1 is absent.

In addition to the interaction with p53, unpublished mass spectrometry data has shown that catalytic components of the nucleosome remodeling and deacetylase complex (NuRD) complex, specifically histone deacetylases 1 and 2 (HDAC1 and 2), interact with MYSM1. They have also been shown to deacetylate p53.

Therefore, we hypothesize that MYSM1 regulates the transcriptional activity of p53 by localizing to DNA with other chromatin modifying factors, such as HDAC1 and HDAC2, and promoting deacetylation directly of p53 or the histones at p53-target promoters such as *Bbc3*/ PUMA and *Cdkn1a*/p21.

Introduction

1. *Hematopoietic Stem Cells*

The components of blood are derived from hematopoietic stem cells (HSCs), which, in adults, are found in the bone marrow, the medulla of the bone[1, 2]. HSCs reside in the yolk sack and the fetal liver during embryonic development and then they accumulate in the bone marrow niche during adulthood[3].These self-renewing cells have the ability to differentiate into all blood cell types, making them multipotent. When these cells divide, they can make more differentiated cells, the multipotent progenitors (MPP), myeloid progenitors (CMP) and lymphoid progenitors (CLP), but also cells of the same type, which maintain the pool of HSCs[4-6]. When stem cells are differentiating to more specialized cell types, their gene expression profiles also change, which further lead to changes in surface proteins that can be detected by flow cytometry[7, 8]. The KLS ($Kit^+Sca1^+Lineage^-$) compartment consists of the long term-HSCs ($Flt3^-CD34^-$), the short term-HSCs ($Flt3^-CD34^+$) and the MPP ($Flt3^+CD34^-$) population[9]. The CMPs give rise to the erythrocytes, platelets, granulocytes and monocytes. The CLPs give rise to T and B cells (Fig.1)

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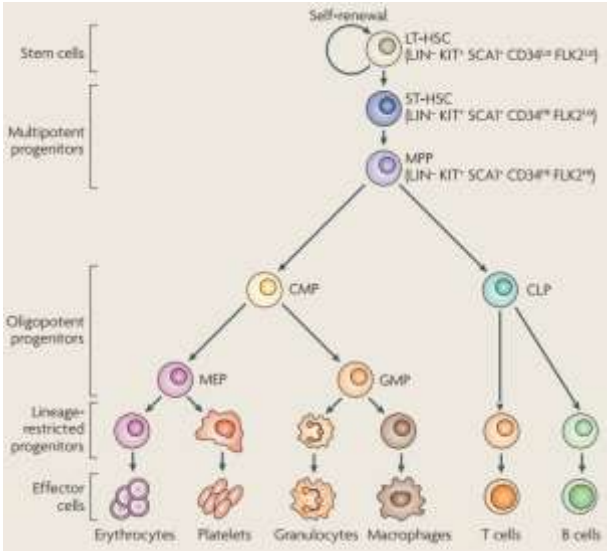

Figure 1[9]: The differentiation of hematopoietic stem cells. A small population of self-renewing, multipotent stem cells gives rise to specialized cells of different blood cell lineages.

The major experiments for this study were done using Ba/F3 cells, which are an interleukin-3 dependent pre-pro B cell line derived from mice. In mammals, B cell development occurs in the bone marrow[10, 11]. It requires the rearrangement of the variable, diversity, and joining gene segments of the immunoglobulin (Ig) heavy and light chain loci. This rearrangement leads to an array of different antigen-binding domains, which allows for greater diversity in antigen recognition. The rearrangements are hallmarks of the different checkpoints in B cell development. In addition to these checkpoints, there are also distinct transcription factors that are present at particular stages of B-cell development[12]. Most importantly, E2A, EBF, and Pax5 are needed for B-lineage differentiation and commitment and PU.1 is needed for functional lymphoid lineage priming[13, 14]. The multipotent progenitors give rise to the CLPs, which further differentiate into pro-B and then pre-B cells[15, 16]. Pre-B cells give rise to immature B cells that migrate to the spleen and other secondary lymphoid organs and differentiate into mature B cells [16, 17]. In the final stages, cells further differentiate into plasma cells, which can secrete antibodies[17].

2. *Epigenetic Regulation*

DNA is the basic template for inheritance of traits from parents to offspring. The approximately 3000 megabases of DNA in the human genome are packaged into chromatin inside the nucleus[18, 19]. Nucleosomes are the basic unit of chromatin with 147 base pairs of DNA wrapped around an octomeric histone core[18, 19]. The histone core is made of two copies each of histones H2A, H2B, H3 and H4 and keeps the DNA in a constrained and compact state (Fig. 2).

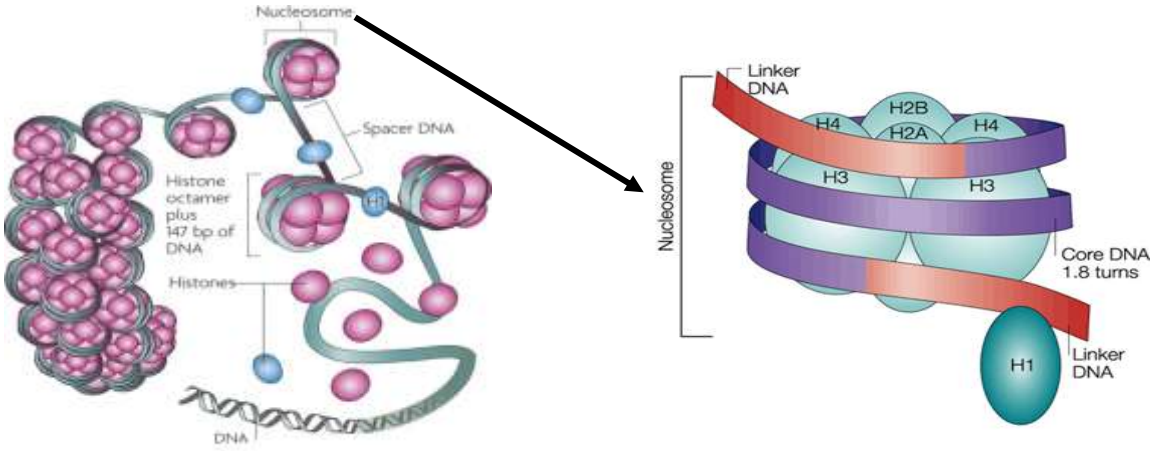

Figure 2[20]: The basic structure of a nucleosome. It is an octamer made up of the core histones H2A, H2B, H3 and H4. Nucleosomes are stringed together via linker DNA that is associated to histone H1[18, 19].

Epigenetic regulation occurs via DNA methylation, histone modifications, chromatin remodelling, and regulation of gene expression by noncoding RNAs[21-25]. DNA methylation has been shown to interfere with the accessibility of DNA to transcription factors that are important for activation [21, 26]. Chromatin remodeling, via ATP hydrolysis, changes the location and conformation of nucleosomes, which can increase or decrease the accessibility of activation marks[25]. Additionally, gene expression can be regulated by miRNAs, which anneal to the 3'untranslated region of cognate mRNAs leading to mRNA instability and/or the inhibition of translation[21].

2.1 *Histone modifications*

According to the histone code hypothesis, biological functions associated with specific regions of the genome are dependent on precise modifications or groups of modifications on histones[22, 27]. Acetylation, methylation, phosphorylation, sumoylation and ubiquitination can either lead to transcriptional activation or repression depending on the site of modification[22]. Normally, acetylation is associated with activation however methylation can lead to one or the other. Marks of activation include H3K4me and H3K4me3, while H3K27me3 and H3K9me3 are marks of repression[22, 25]. Permissive marks are those which can either be activating or repressive depending on other modifications (Fig. 3)[22-24].

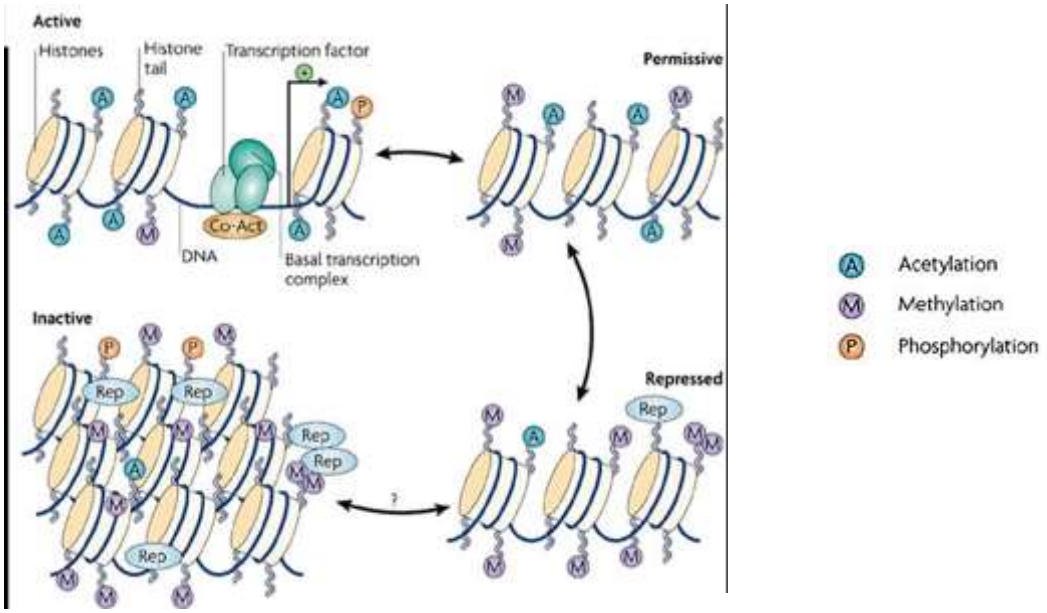

Figure 3[28]: Active, repressive, and permissive histone modifications. Activating marks of acetylation, methylation and phosphorylation (amongst others) allow chromatin to be in an open state while repressive methylation and phosphorylation closes chromatin and does not allow transcription to proceed.

2.2 *Ubiquitination*

Ubiquitination involves the covalent addition of ubiquitin, a polypeptide made up of 76 amino acids to target proteins or histone tails, which can change their function, stability and localization[29, 30]. It is a process that requires three classes of enzymes: E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin-ligating) [31]. Monoubiquitination of histone H2A and H2B is generally associated with transcriptional silencing and activation, respectively. H2A was the first protein shown to be ubiquitinated at the K119 residue and is the most abundantly ubiquitinated protein in the mammalian nucleus[32, 33]. H2Aub is generally associated with repression in transcriptional regulation[34]. Though the exact mechanisms of repression are not completely understood, several studies have shown a halt in RNA polymerase II at H2Aub-enriched promoters[34]. Alternatively, H2Bub has been shown to be important for nucleosome reassembly and chromatin structure restoration, which lead to a change in the kinetic properties of elongating RNA polymerase II[35].

K63-polyubiquitination is important for regulation of intracellular events such as DNA repair, endocytosis, signalling and trafficking[36-38]. Specifically, K63-polyubiquitination of histones H2A at K13 and K15 is an early mark of DNA damage and is important in the recruitment of repair proteins to DNA damage foci. Alternatively, K48, and potentially K11-polyubiquitination, is a mark for degradation via proteosomal targeting[36, 39].

2.3 *Deubiquitinating proteins*

In order to counteract the effects of ubiquitination, proteases known as deubiquitinases work to remove and recycle ubiquitin groups[40, 41]. There are approximately 95 genes in the human genome that encode deubiquitinase proteins[41]. These proteins are a part of five families including ubiquitin specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), otubain proteases (OTUs), Machado-Joseph disease proteases (MJDPs), and the JAB1/MPN/Mov34 metalloenzyme (JAMM) domain zinc-dependent metalloproteases (Fig. 4)[40].

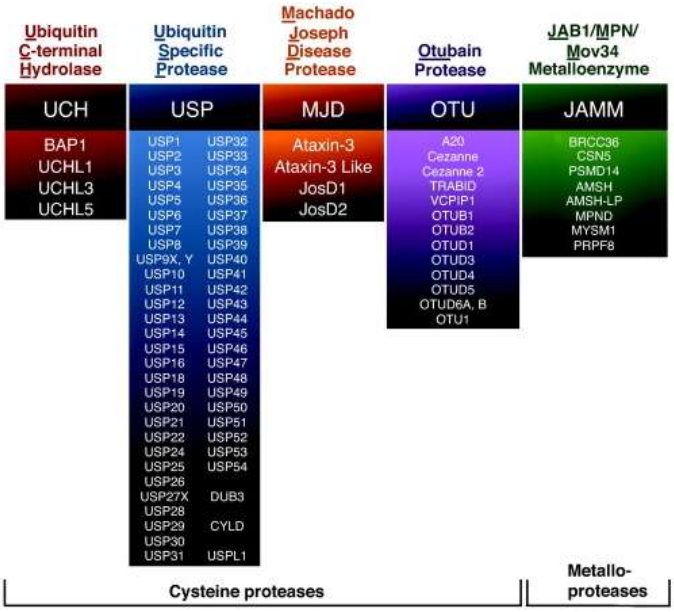

Figure 4[42]: Different families of deubiquitinating enzymes. The UCHs, USPs, MJDPs and OTUs are cysteine proteases while the JAMMs are metalloproteases[41].

The first four families are made up of cysteine proteases while the last consists of metalloproteases[29]. Deubiquitination is important for cell cycle regulation, gene expression, DNA repair and a number of other cellular processes. Of relevance to this project are the JAMM domain metalloproteases, which coordinate histidine, aspartate and serine residues with zinc ions[43]. This causes an activation of water molecules, which allows them to target the isopeptide bond leading to the dissociation of ubiquitin.

3. *Myb-Like, SWIRM and MPN Domains 1 (MYSM1)*

The MYSM1 protein is a metalloprotease that was originally identified as a deubiquitinase for monoubiquitinated histone H2A. MYSM1 contains three domains: JAMM, SANT, and SWIRM domains (Fig. 5)[44].

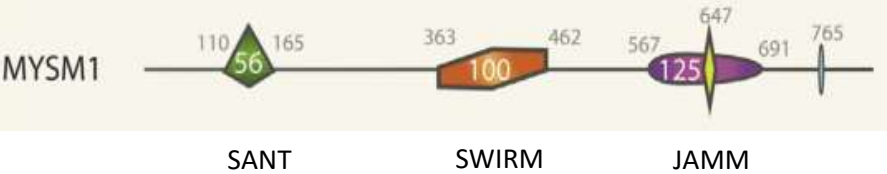

Figure 5[44]: The structure of MYSM1. This deubiquitinase contains 3 domains. The SANT and SWIRM domains are for DNA binding while the JAMM domain is important for the catalytic activity of the protein.

The JAMM metalloenzyme domain has an intrinsic metalloprotease activity, which is necessary for the catalytic activity of isopeptide bond hydrolysis[45-48]. The SANT domain binds specifically to DNA and histones[49]. Finally, the SWIRM domain is important for the interactions with histone H3 and the linker DNA between histones[50-52].

3.1 *Mouse model of Mysm1 deficiency*

When *Mysm1* is deleted in mice, they have physiological and hematological defects that are rescued when p53 is deleted as well[53]. *Mysm1*-knockout mice (*Mysm1*$^{tm1a/tm1a}$) have severe defects such as hind limb abnormalities, lack of tail and reduced body size and weight (Fig. 6A)[53].

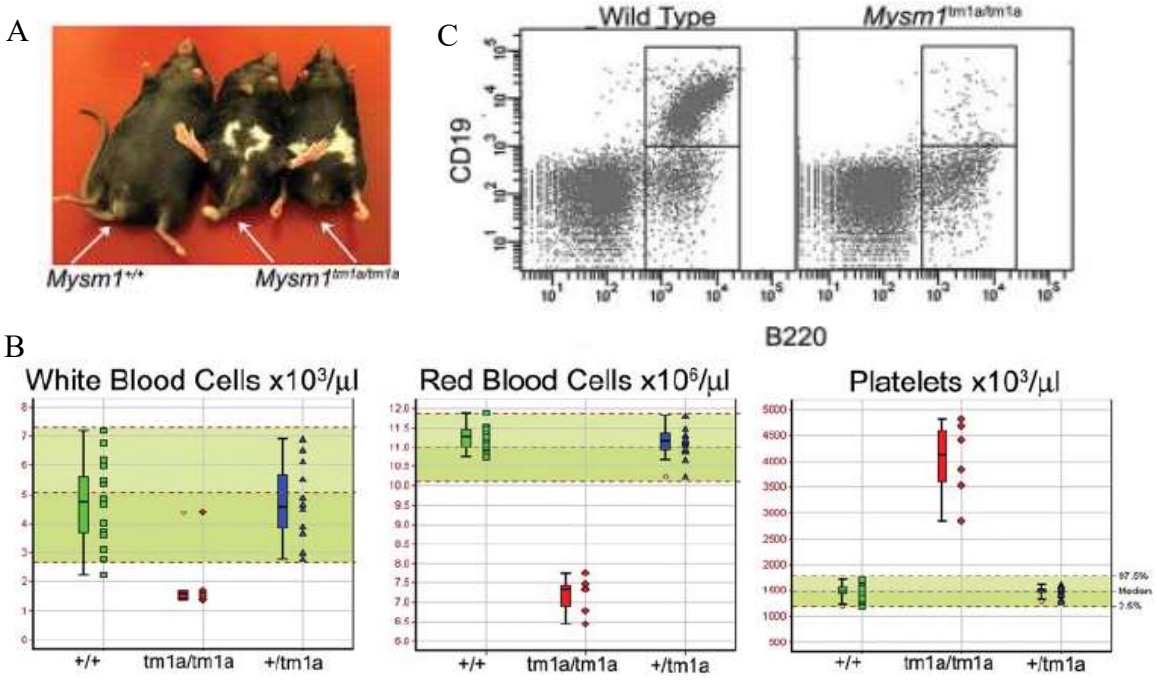

Figure 6[53]: Features of *Mysm1*$^{tm1a/tm1a}$ mice in comparison to *Mysm1*$^{+/+}$. A) Physically, the knock out mice are smaller in size, have white belly spots and almost no tails. B) In terms of their hematopoesis, the knockout mice have lower white and red blood cells counts and higher platelet counts than the wild type counterparts. C) There are a lower number of B lymphocytes from early pre-pro B cell stage (gated on CD19^{+}B220^{+}).

Mysm1-knockout mice also have features of bone marrow failure such as reduced white and red blood cell counts, hematocrit and blood hemoglobin content (Fig. 6B)[53]. There was also a severe reduction in the B lymphocytes starting from the early pre-pro B cell stage and defects in T-cell development as seen by flow cytometry (Fig. 6C)[53]. There was a loss of HSC quiescence and

function in addition to elevated apoptosis of MPPs and other hematopoietic progenitors[53]. These findings as well as the functional failure of *Mysm1*$^{tm1a/tm1a}$ HSCs in bone marrow transplantation assays lead to the conclusion that *Mysm1* is essential for normal progression of hematopoiesis[53]. Indeed, it was confirmed that *Mysm1* is expressed in hematopoietic stem cells and the earliest hematopoietic progenitors[53]. Importantly, it was also seen that when *Mysm1* is absent, there is an upregulation in p53 in the early hematopoietic progenitors[53].

3.2 *Mysm1*$^{-/-}$*p53*$^{-/-}$ *mice have a rescued hematopoietic phenotype*

Double-knockout mice for *Mysm1* and *p53* show a complete rescue of the *Mysm1*$^{-/-}$ phenotype[54]. The MPP, CLP and CMP hematopoietic progenitor cell populations, and mature hematopoietic cells such as B cells, T cells, and NK cells were at levels similar to their wildtype counterparts[54]. These mice also had a physical restoration of their phenotype as they were normal in length and weight with full-sized tails[54]. Upon doing competitive bone marrow transplantation experiments, it was seen that the bone marrow from *Mysm1*$^{-/-}$*p53*$^{+/+}$ was not able to compete with the wild-type bone marrow, but when *p53* was either partially or completely inactivated with complete *Mysm1* inactivation, there was a rescue[54]. It was also seen that the HSC and MPP cell pools in the recipient mice were derived from double-knockout cells, and not *Mysm1* knock out cells[54]. Overall, the loss of p53 rescues the hematopoietic stem cell and progenitor cell functions. These data show that the deubiquitinase *Mysm1* antagonizes p53 in hematopoiesis.

3.3 *MYSM1 interacts with p53*

In order to understand what was occurring at the molecular level, FLAG-MYSM1 was expressed and pulled down in Ba/F3 hematopoietic progenitor cells, and p53 was seen in the immunoprecipitates and vice versa (Fig. 7A)[55]. These results show that MYSM1 and p53 do indeed interact. They are both transcriptional regulators that bind chromatin and for this reason chromatin immunoprecipitation (ChIP) studies were done in order to determine if MYSM1 gets recruited to known p53 binding sites at the promoters of p53-regulated stress response genes (*Bbc3*/ PUMA and *Cdkn1a*/p21). Indeed, there was MYSM1 binding at these sites, which was enhanced when the cells were irradiated (3Gy, X-rays) to induce the p53 stress response (Fig. 7B) [55]. In addition to this, Ba/F3 cells with an shRNA *Mysm1* knockdown showed an increase in p53 recruitment at the *Bbc3* and *Cdkn1a* promoters, which implies a negative correlation between MYSM1 and p53 (Fig. 7C)[55]. Interestingly, the lack of MYSM1 also led to an increase of histone H3K27 acetylation at these promoters, which is a mark of transcriptional activation (Fig. 7D) [55]. In the current proposed model, it is suggested that when MYSM1 is present, it antagonizes histone acetylation and p53 recruitment, however when MYSM1 is absent, transcription at p53 target promoters can proceed as there is no inhibition.

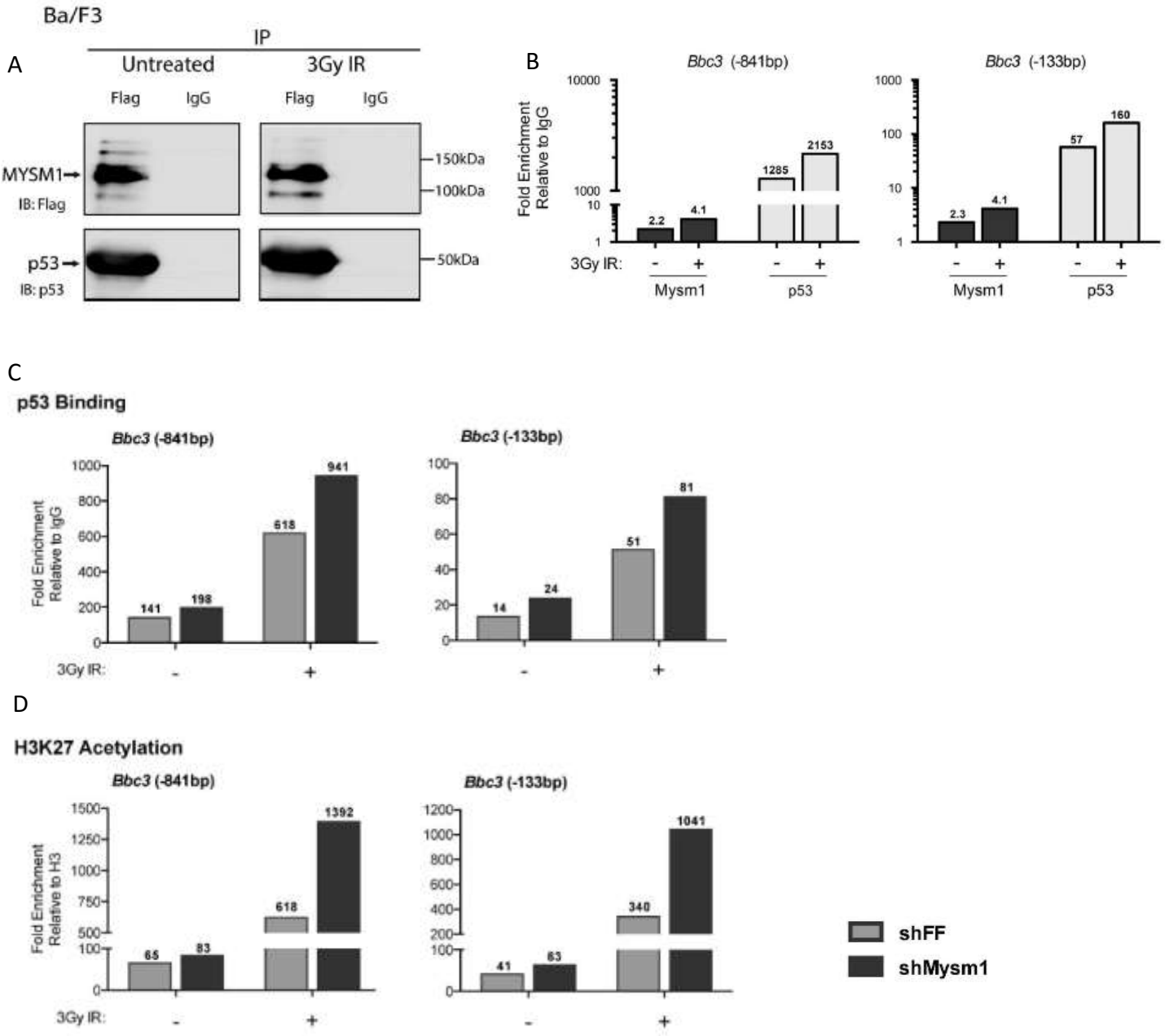

Figure 7[55]: Interaction of MYSM1 and p53. A) When endogenously expressed FLAG-MYSM1 was pulled down in Ba/F3 cells, p53 was seen in the immunoprecipitates. B) There is MYSM1 binding at *Bbc3* (shown here) and *Cdkn1a* promoters at known p53 binding sites. It is amplified when the cells are irradiated. C) Ba/F3 cells with a shRNA *Mysm1* knockdown show an increase in p53 recruitment in proximity of the *Bbc3* (shown here) and *Cdkn1a* promoters. D) The lack of MYSM1 led to an increase of H3K27 acetylation at *Bbc3* (shown here) and *Cdkn1a* promoters.

3.4 *Additional roles for* MYSM1

In addition to hematopoietic regulation through interactions with p53, a loss of MYSM1 has also been shown to lead to a decreased recruitment of Gata2 and Runx1 transcription factors to the *Gfi1* locus, encoding an essential hematopoietic transcriptional regulator[56]. H3K27me3, H2AK119ub, and stalled RNA polymerase II were also detected at this locus in *Mysm1*-knockout cells, which are marks of repression[56]. The authors of this study suggested that MYSM1 causes the de-repression of *Gfi1,* which maintains HSC quiescence, and therefore *Mysm1*-deficient HSCs exhibit loss of quiescence and increased cell cycling[56]. By using ChIP, another group showed that MYSM1 is essential for B cell maturation[57]. *Mysm1*-deficient CLPs and Pro-B cell showed a decrease in the expression of *Ebf1* and *Pax5* genes, which encode B-cell commitment transcription factors[57]. When *Mysm1*-deficient bone marrow cells were transduced with Ebf1-expressing vectors, there was a rescue in their ability to produce mature B cells, which suggests that MYSM1 regulates B cell development by promoting Ebf1 expression[57]. Furthermore, MYSM1 has been shown to be important for natural killer (NK) cell maturation as mice deficient in *Mysm1* have severely impaired NK cells development[58]. Flow cytometry analysis showed an accumulation of NK progenitors and immature NK cells and a deficiency in mature NK cells in *Mysm1*-knockout mice. A transcription factor that is known to be important for NK cell development, inhibitor of DNA-binding protein 2, is also decreased in expression in *Mysm1* deficient hematopoietic progenitors[58]. Another study showed that *Mysm1* deficient mice had lower bone mass than control mice and that their mesenchymal stem cells showed increased differentiation and adipogenesis[59]. Using isolated WT and *Mysm1*$^{-/-}$ pro-osteoblasts that were subjected to specific induction medium and then stained using Alizarin red S (stains calcium nodules in osteogenic induction), MYSM1 was shown to be important for differentiation into

adipocytes or osteoblasts[59]. Overall, MYSM1 has been shown to have important effects in different lineages of hematopoiesis and in the cells of the hematopoietic bone marrow niche.

In addition to a role in the transcriptional regulation of hematopoiesis, MYSM1 has also been shown to be important in the regulation of immune and inflammatory responses[60]. Normally, MYSM1 is localized to the nucleus, however, when there is the presence of a microbe, it can accumulate for short periods of time in the cytoplasm. The SWIRM and MPN domains of MYSM1 interact with TRAF3 and TRAF6 signalling complexes, promote their deubiquitination, and therefore supresses inflammatory response and type-I interferon production. The lack of proper regulation can lead to a self-destructive immune response[60].

3.5 *MYSM1 in humans*

Mutations in the *MYSM1* gene have also been shown to have implications in humans. Alsultan *et al* (2013) studied 2 siblings with inherited bone marrow failure syndromes from a consanguineous family who had a similar hematopoietic phenotype to the *Mysm1* deficient mice generated previously by Nijnik *et al* (2012) (i.e. low white blood cell counts, anemia, etc.)[53, 61]. Exome sequencing showed that there is the same single homozygous variant in both patients (*MYSM1:* NM_001085487:exon8.c1168G>T.pE390*) associated with the disease[61]. The mutation leads to a biallelic truncation of *MYSM1,* which is the likely cause of their disease phenotype. More recently, a patient with T-cell lymphopenia, defective hematopoiesis, developmental abnormalities and no B lymphocytes was shown to have a homozygous *MYSM1* missense mutation (c.1967A>G) that affected the catalytic JAMM domain[62]. These studies show that MYSM1 is necessary for proper immune and hematological development in humans, in addition to mice.

4. *Tumor suppressor protein 53*

p53 is a tumor suppressor protein, encoded by the *TP53* gene, that is mutated in most human tumor types[63, 64]. It contains 4 domains: an N-terminal transactivation domain, a central DNA binding domain, a tetramerization domain, and a C-terminal regulatory domain (Fig. 8). Each of these regions undergoes post-translational modifications at different sites that play a role in stabilizing p53 and regulating its function[65]. Mice that have a dominant negative p53 mutation or p53-deletion have an increased susceptibility to tumors[66], while mice with a p53 gain-of-function show protection against tumors[67]. Individuals who only have one functional copy of the *TP53* gene have a rare condition known as the Li-Fraumeni syndrome and have a predisposition to cancer[68].

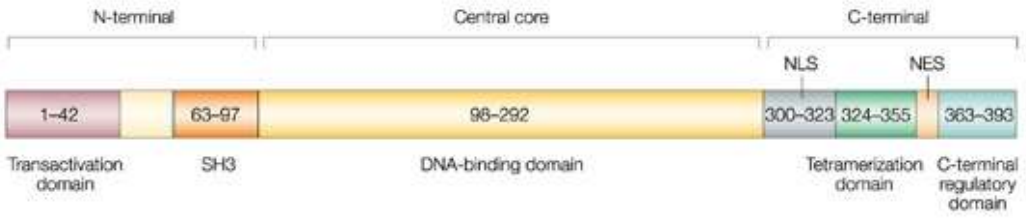

Figure 8: p53 tumor suppressor protein has 4 domains[69].

4.1 *Transcriptional regulation by p53*

p53 regulates the expression of hundreds of genes, which cause cell cycle arrest (ex. *Cdkn1a/*p21), cellular senescence, or apoptosis (ex. *Bbc3/*PUMA) [70-73]. Additionally, p53 has been shown to regulate HSC quiescence, self-renewal and differentiation potentials[74-76]. A well-known target of p53 is $p21^{Waf1/Cip1}$, which gets activated when p53 binds and activates the *Cdkn1a* promoter[70]. It leads to the production of the p21 protein, a cyclin dependent kinase inhibitor that binds the cell division-stimulating protein (cdk2), prohibiting cell cycle progression until necessary. It has been shown that p53 loss of function prevents *Cdkn1a* gene expression, which causes a block in p21 production and allows cell cycle progression to occur[70].

If the cells cannot be repaired, they need to be destroyed and for this, p53 induces pro-apoptotic proteins such as phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1; also known as NOXA) and p53-upregulated modulator of apoptosis (PUMA) [72, 73]. Specifically, once PUMA has been activated, it binds to and inhibits mitochondrial anti-apoptotic proteins such as Bcl-2, which frees Bax and Bak for mitochondrial apoptotic signaling[73]. The pro-apoptotic Bax and Bak cause the permeabilization of the mitochondrial membrane, which allows for the release of cytochrome c. This allows for caspase activation, leading to cell death. Alternatively, p53 can also cause caspase activation through transmembrane proteins via transcriptional activation of apoptotic regulators such as *Dr5*[77].

4.2 *Regulation of p53 function*

Generally, p53 levels are kept low in the cell however, when cells are stressed, there is an increase in order to cause an arrest in growth, to repair DNA, or cause cell death as a final resort (Fig. 9)[78, 79]. The mechanisms involved will be discussed in the following section.

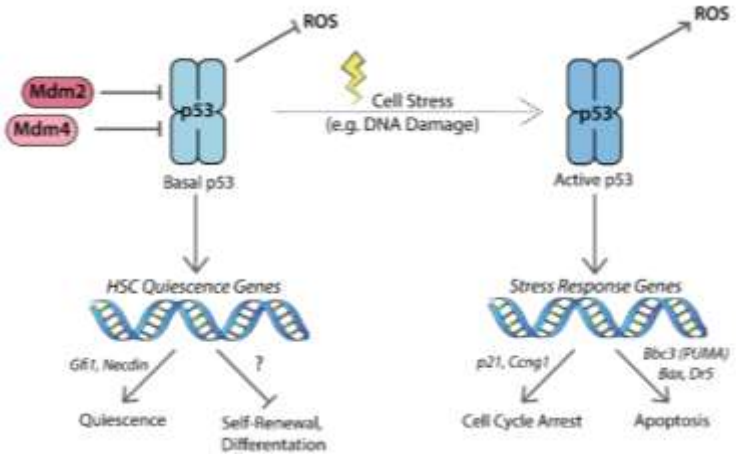

Figure 9: p53 Regulation. The levels of p53 are kept under control at basal levels by E3 ubiquitin ligases Mdm2 and Mdm4. Cell stress causes an increase in p53 levels needed for processes such as cell cycle arrest, DNA repair and apoptosis (figure prepared by Jad I. Belle).

Though p53 is an important tumor suppressor, excessive levels of the protein can lead to cellular aging[80-82]. Mdm2, an E3 ubiquitin ligase, can trigger the degradation of unnecessary p53 via the ubiquitin system[83-85]. In addition to being regulated by Mdm2, p53 also controls the expression of the *Mdm2* gene[83]. When p53 gets phosphorylated at Ser15 (mouse Ser18),or Ser20 (mouse Ser23) via a stress response activated protein kinase such as ATM or ATR, Mdm2 or Mdm4 can no longer bind to it[86]. This allows p53 to bind transcriptional coactivators such as histone acetyl transferases p300 and CREB-binding protein that transfer acetyl groups to lysines that are normally targeted by Mdm2[86]. Specifically, p53 gets acetylated at C-terminal lysine residues that are usually targeted for ubiquitination (human K370, 372, 373, 381, 382, 386 corresponding to mouse K367, K369, K370, K378, K379, K383), by these acetyltransferase complexes[87]. K320 in humans (mouse K317), a site outside the C-terminus, also gets acetylated

by pCAF[88]. Mutation of this lysine to arginine led to increased proapoptotic gene expression and apoptosis in the thymus and spleen after stress caused by irradiation[89]. Increased p53 levels also mean that there is a greater production of Mdm2 and 4, however posttranslational modifications prohibit the binding between p53 and Mdm2 and 4[86]. When the stress response has ceased and the damage has been repaired, the kinases no longer phosphorylate p53, which allows the accumulated Mdm2 to bind and target it for degradation via ubiquitination[86]. Additionally, different phosphatases such as WIP1 and DUSP26 have been shown to reverse the effects of cell cycle arrest by dephosphorylating p53[90].

5. *MYSM1 and the NuRD complex*

In addition to the interaction with p53, unpublished mass spectrometry data on mouse embryonic stem cells has shown that MYSM1 interacts with different components of the nucleosome remodeling and deacetylase complex (NuRD) [91]. NuRD is a chromatin remodeling complex that is expressed in most tissues and plays diverse roles in transcriptional regulation, cell cycle progression, and stability of the genome[92]. In terms of the structure, NuRD has 6 different subunits (Fig. 10)[93].

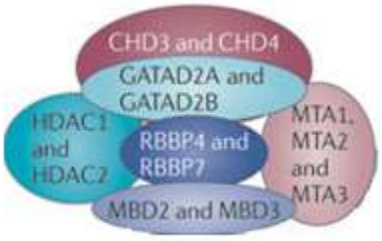

Figure 10[93]: The NuRD complex is made up of 6 subunits.

The chromodomain-helicase-DNA-binding proteins 3 and 4 (CHD3/4) and the histone deacetylases 1 and 2 (HDAC1/2) are the catalytic subunits of the complex [94]. CHD3 and CHD4 are chromatin remodelers while HDAC1 and HDAC2, as the names suggest, have deacetylation activity[95, 96]. From the remaining subunits, the methyl-CpG-binding domain proteins (MBD2 and

3) are for associations with methylated DNA and the metastasis-associated gene proteins (MTA1, 2, and 3) are for associations with transcription factors and have been shown to be important in targeting the NuRD complex to specific genomic locations[97]. Finally, the retinoblastoma-binding proteins 4 and 7 (RBBP4/7) and the GATA Binding Protein 2 (GATAD2A/2B) proteins have been shown to be a part of the NuRD complex in a structural capacity as well as for histone tail binding[98-100]. These subunits are not specific to the NuRD and can associate with other complexes as well (coREST and SIN3 complexes, for example) [101].

5.1 *Histone acetyl transferases (HATs) and Histone Deacetylases (HDACs)*

There are two well-known acetylation targets when it comes to post translational modifications. First, proteins can be acetylated when there is the addition of an acetyl group to the ε-amino group of lysine residues (N^{ε}-acetylation) [102]. Alternatively, N-terminal acetylation is associated with histone proteins[103, 104]. In both cases, acetyl groups get transferred to their targets via histone acetyl transferases (HATs) and get removed by histone deacetylases (HDACs). Generally, when histones are acetylated, the chromatin is in a relaxed state, leading to transcriptional activation and when histones are deacetylated, the chromatin is in a condensed state, leading to transcriptional silencing[105]. Acetylation reduces the positive change on the histone tails[106]. The negative charge of the acetyl groups repels the DNA phosphate backbone, which is also negatively charged. This change leads to a disruption in the association of the nucleosome components, which allows the DNA to be more accessible by transcription factors, leading to activation[106]. When deacetylation occurs, the DNA essentially wraps around the histone cores tightly, which means transcription factors cannot access the DNA as readily, leading to repression[107-109]. Furthermore, once acetylated, the lysine residues can be recognized by specific motifs called bromodomains, which are present in nucleosome remodeling proteins

that are important for transcriptional initiation[110]. The modification targets the proteins to the specific promoter for gene expression regulation. Examples of bromodomain containing proteins include pCAF and the CREB-binding protein[110].

5.2 *Biochemistry of HDACs*

There are four classes of HDACs in humans: Class I, II, III, and IV[111]. Class I, which includes HDAC1 and 2 of the NuRD complex, class II and class IV are a part of the classical family that are similar in sequence and that require Zn^{2+} for deacetylase activity[111]. Class III deacetylases make up the silent information regulator 2 (Sir2) - related protein (sirtuin) family. The deacetylases that are a part of this family are not similar in sequence to the classical deacetylases and they require NAD^{+} and not Zn^{2+} for full activity[111]. Usually, HATs and HDACs exist in large multi-protein complexes [112, 113]. As is the case with the NuRD complex, in addition to the enzymatic proteins, there are typically also structural proteins that are necessary for regulating the complex.

In terms of HDAC1 and 2, there has been debate about whether or not both are necessary for proper functioning of the complexes that they are a part of. Deletion of HDAC1 in mice leads to embryonic lethality while the deletion of HDAC2 results in varying phenotypes in different studies[114]. Some mice with a HDAC2 deletion were viable with reduced body weight, some had cardiac myopathies while others benefitted from enhanced synapse formation, learning and memory[115-118]. These varying phenotypes may be due to different genetic backgrounds of the mice. When HDAC1 or HDAC2 are conditionally knocked out in mice in the hematopoietic system and liver, there is no hematopoietic phenotype, which implies that their function is redundant and one takes over while the other is not functional[117]. When both are conditionally knocked out, the mice have severe anemia and thrombocytopenia[117].

5.3 *Importance of the NuRD complex*

Using CHD4 conditional knockout mice, NuRD was shown to be important in hematopoiesis, specifically the differentiation of hematopoietic stem cells into the lymphoid and myeloid lineage cells as well as the maintenance of these stem cells[119]. Additionally, since the deletion of specific components of the complex can lead to embryonic lethality, it would seem that the NuRD complex is important for embryonic viability. Conditional inactivation of the ATPase domain of CHD4 in thymocytes also showed the importance of NuRD in the transcriptional regulation of T lymphocyte development[120]. Interestingly, NuRD has been shown to deacetylate p53, which inactivates it[121]. When p53 is not active, downstream target p21 is not expressed and cannot cause a block in cell cycle progression. When different subunits of the complex were manipulated by RNA interference, there was a blockade in the G1/S phase transition as well as an increase in p21[121]. Additionally, a chromatin localization screen showed that when there are double-stranded DNA breaks due to damage, NuRD and other proteins get recruited by poly (ADP ribose) polymerase (PARP) through poly (ADP ribose) chains for repair[121, 122]. In this scenario, NuRD also helps in the recruitment of other repair proteins and promotes transcriptional silencing so that the cells can be repaired before resuming regular processes[122]. When CHD4 is depleted, there is an increase of unrepaired DNA breaks[121]. In addition to developmental and repair processes, NuRD has also been implicated in cancer biology, specifically in epithelial-to-mesenchymal transition, which increases cell motility and is a part of metastasis[123]. The MTA family subunits of the NuRD complex are thought to promote this progression[123].

5.4 *Inhibition of HDACs*

The inhibition of HDACs has been shown to lead to cell cycle arrest, apoptosis and cellular senescence[124]. Specifically, the knockdown of HDAC2 has been shown to inhibit proliferation and induce senescence. When HDACs are not active, p53 can be stabilized due to acetylation at Lys 320, 373 and 382[125, 126]. This allows for the upregulation of downstream targets previously discussed such as p21, as well as p53 regulators like Mdm2. It has been noted however, that the treatment of patients with HDAC inhibitors leads to undesirable hematological side effects such as anemia and thrombocytopenia but the reasons for this response are not well understood[127]. Though HDAC inhibitors can target classes, they cannot yet be made to target specific HDACs, which may be one reason for unwanted problems associated with treatment. Additionally, there could also be off-target effects on non-HDAC proteins as a result of HDAC inhibition[127]. Though the exact method of action of these inhibitors is not yet completely understood, they serve as a promising method of cancer treatment. For the time being, inhibitors have been used for cutaneous (Vorinostat), peripheral (Romidepsin and Belinostat) and relapsed (Belinostat) T-Cell lymphomas in clinical trials[128-131]. Vorinostat and Belinostat inhibit Class I, II and IV HDACs while Romidepsin inhibits class I and II HDACs. Each of these inhibitors has shown promising results in the treatment of cancer in clinical trials.

6. *Rationale*

We are suggesting that the chromatin interacting deubiquitinase MYSM1 regulates hematopoiesis and lymphocyte development via p53-dependent mechanisms. This is based on studies showing interactions between MYSM1 and p53 proteins, as well as increased recruitment of p53 and activating histone acetylation marks at known p53-target gene promoters when MYSM1 is absent. Furthermore, unpublished mass spectrometry data suggests that MYSM1 interacts with HDACs 1/2, which are known to act as negative regulators of p53. Therefore, we hypothesize that MYSM1 regulates the transcriptional activity of p53 by localizing to DNA with other chromatin modifying factors, such as HDAC1 and HDAC2, and promoting deacetylation directly of p53 or the histones at p53-target promoters such as *Bbc3*/PUMA and *Cdkn1a*/p21.

Materials and Methods

Tissue culture and transfection

H1299 cells were maintained in RPMI-1640 (Life Technologies) with 10% FCS and 2 mM L-Glutamine (Wisent). Cells were passaged every 2–3 days and all transfections were performed within 10 passages from thawing. The cells were transiently transfected using Lipofectamine 2000 (Life Technologies) with pcDNA3.1(+) vector encoding N-terminal HA-tagged and C-terminal 6x His-tagged human MYSM1 (Life Technologies) and pcDNA3.1(+) vector encoding N-terminal FLAG-tagged HDAC1, HDAC2 or MTA2. Ba/F3 cells were maintained in RPMI-1640 (Life Technologies) with 10% FCS, 5% WEHI conditioned media, 2 mM L-Glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin and 2µg/mL of puromycin (Wisent). The cells were maintained at 0.5–2 × 10^6 cells/ml at all times.

shRNA Knockdown

The Mysm1 transcript was initially targeted by six different shRNA sequences. Oligonucleotides were PCR-amplified and cloned into the pMSCV-mir30-PIG (MLP) vector[132]. For off-target knockdown controls, the MLP-shFF was used. The retroviruses were produced in Pheonix cells[133] (ATCC, Manassas, VA, USA) and then Ba/F3 cells were infected, selected and maintained in 2µg/mL of puromycin (Wisent). The following shRNA was chosen: 5′-TGCTGTTGACAGTGAGCGACCGGGAAATGATGAAAGTACATAGTGAAGCCACAGAT GTATGTACTTTCATCATTTCCCGGCTGCCTACTGCCTCGGA-3′ based on knockdown efficiency.

Ba/F3 irradiation assays

Independently infected shFF and shMysm1 lines were plated at 1×10^6 cells/ml in fresh media without IL-3 at the beginning of each experiment. As IL-3 has been shown to block apoptotic programs in this cell line[134, 135] it was excluded from the experiments to avoid masking of p53-mediated stress responses. Cells were irradiated with 3 Gy in a RS2000 irradiator (Rad Source) at indicated time points and always harvested alongside untreated cells to control for endogenous stress.

ChIP and qPCR

ChIP was performed as described previously[136] with minor modifications. Briefly, cells were fixed by addition of formaldehyde in the culture media to a final concentration of 1%, and were incubated for 11 min at room temperature, followed by addition of 0.125 M of glycine to stop fixation. Nuclei were then extracted with 5 min lysis in 0.25% Triton buffer (10 mM Tris-HCl pH 8, 10 mM EDTA, 0.5 mM EGTA), followed by 30 min lysis in 200 mM NaCl buffer (10 mM Tris-HCl pH 8, 1 mM EDTA, 0.5 mM EGTA). Nuclei from 5×10^6 cells were resuspended in sonication buffer (10 mM Tris pH 8). Six washes were performed with low stringency buffers. Samples were de-crosslinked by overnight incubation at 65°C in 1% SDS buffer (50 mM Tris pH 8, 10 mM EDTA), and following RNaseA and Protienase K enzymatic treatments, ChIP DNA was purified using the Qiaquick PCR cleanup kit (Qiagen, Venlo, Netherlands). ChIP enrichment was quantified using Real Time qPCR analysis (Primer sequences in Table 1). Real-time qPCR analysis was done on an Applied Biosystems StepOnePlus instrument with Power SYBR Master Mix (Applied Biosystems). All CT values were normalized to those of the pro-opiomelanocortin (Pomc) gene, which serves as a negative binding region. Enrichment was calculated relative to input for transcription factors.

Co-immunoprecipitation and Western blotting

For co-immunoprecipitation, H1299 cells were lysed in B450 buffer (25 mM Tris pH 7.5, 2 mM EDTA, 450 mM NaCl, 0.2% NP40, 5% glycerol) 48 h after transfection. Samples were dounce homogenized and cleared using centrifugation. Cleared lysates were diluted 1/3 to 150 mM NaCl and 0.1% NP40 and their protein content quantified with the BCA assay (Thermo Scientific, Waltham, MA, USA). Antibody-conjugated Dynabeads Protein G (Invitrogen, Life Technologies) was prepared by incubation of 40 µl bead slurry with 3 µg of FLAG M2 (Sigma), His or control IgG (Santa Cruz) for at least 4 h. Immunoprecipitation of the protein lysate with antibody-conjugated Dynabeads was performed with rotation at 4°C for 2 h. 500 µg total protein was used. After four washes with IP buffer (25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% NP40), protein complexes were eluted in 1 × SDS sample buffer with boiling for 10 min at 95°C. Eluates were then separated from the beads and reduced by addition of 50 mM DTT, followed by heating for further 10 min at 95 °C. For Western blot analysis, cells were lysed in a modified RIPA buffer supplemented with 1mM DTT and protease and phosphatase inhibitors (Thermo Scientific). Protein concentration was assessed using the BCA assay (Thermo Scientific), and samples were prepared by boiling in Laemmli buffer.

Table 1. ChIP-qPCR Primer Sequences.

Region	Forward Sequence	Reverse Sequence
Bbc3 841bp upstream (Chr7: 16894091)	TCCAGTGCCAGATGGGTATTA	CTGAGACAGGTTCCTGCTAAATG
Bbc3 133bp upstream (Chr7: 16894799)	TGGTCTGACTTTGTGTCCCT	GCTTGACACACTGACACACT
p21 short TSS (Chr17: 29231448)	CCAAAGCGTGAGAATGAAGCTC	GCTCTGCGCTAAGCTCTAGATA
p21 long TSS (Chr17: 29227882)	GAGACCAGCAGCAAAATCG	CAGCCCCACCTCTTCAATTC
POMC (Chr12:3954598)	AGGCAGATGGACGCACATAGGTAA	TCCACTTAGAACTGGACAGAGGCT

Table 2. Antibodies used in ChIP experiments.

Antigen	ID	Supplier	Origin
HDAC1	ab7028	Abcam®	Rabbit polyclonal
HDAC2	ab7029	Abcam®	Rabbit polyclonal
p53 (K370Ac)	2570S	Cell Signaling Technology®	Rabbit polyclonal

Table 3. Antibodies used in Western Blotting Experiments.

Antigen	ID	Supplier	Origin
MYSM1	NA	Produced	Rabbit polyclonal
FLAG	F1804 (M2)	Sigma-Aldrich®	Mouse monoclonal
HA	MMS-101P	Covance®	Mouse monoclonal
His	SAB1306084	Sigma-Aldrich®	Rabbit polyclonal
p53	2524S (1C12)	Cell Signaling Technology®	Mouse monoclonal
p53 (K370Ac)	2570S	Cell Signaling Technology®	Rabbit polyclonal
p53 (K320Ac)	SAB4503014	Sigma-Aldrich®	Rabbit polyclonal
β-Actin	8457S	Cell Signaling Technology®	Rabbit monoclonal

Results

MYSM1 interacts with HDAC2, and potentially HDAC1 and MTA2

Previously unpublished mass spectrometry analysis of mouse embryonic stem cells has shown that different components of the NuRD complex interact with MYSM1[91]. To verify the interaction of the human counterparts of these proteins, co-transfection experiments were done using H1299 cells. This cancerous cell line is derived from the human lung and has a homozygous deletion of the p53 protein[137]. This cell line allows for the efficient expression of human proteins to understand their interactions without the interference of the p53 protein. Human MYSM1 was subcloned into the pcDNA3.1 (+) vector. The cells were co-transfected with human HA- and 6x His- tagged MYSM1 and FLAG-tagged HDAC1, HDAC2, or MTA2. As previously mentioned, HDAC1, HDAC2, and MTA2 are components of the NuRD complex. The MYSM1 protein was detected in the HDAC2 immunoprecipitates (Fig. 11A). The HDAC2 protein was detected when MYSM1 was immunoprecipitated (Fig. 11B). MYSM1 also seemed to be present in the HDAC1 and MTA2 immunoprecitipates, however there was not a distinct band at approximately 90 kDA, as was the case for the HDAC2 immunoprecipitation (Fig. 11C, D). Instead, there were two bands, one at the correct location and one slightly above it. This data indicates an interaction between MYSM1 and HDAC2, and a potential interaction between MYSM1 and HDAC1 or MTA2.

Endogenous immunoprecipitation studies were done using a Ba/F3 hematopoietic progenitor cell line expressing 3x FLAG-tagged mouse MYSM1. The interaction between MYSM1 and HDAC1 or HDAC2 was inconclusive in this case as there was heavy and light chain interference close to the estimated HDAC band sizes of approximately 55kDA in both test and control immunoprecipitates (data not shown).

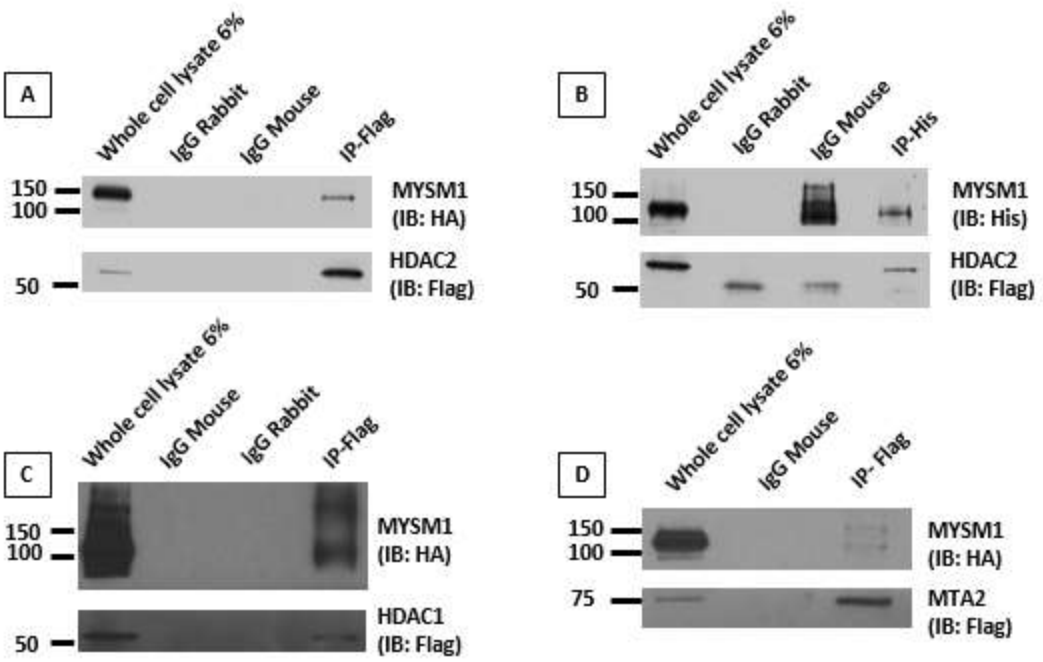

Figure 11: MYSM1 interacts with HDAC2, and potentially HDAC1 and MTA2.

H1299 cells were co-transfected with equal amounts of HA/6xHis- MYSM1 and FLAG-HDAC1, FLAG-HDAC2 or FLAG-MTA2 expressing vectors. At 48hrs, cell lysates were subjected to immunoprecipitation with anti-FLAG (M2) antibody or with anti-His antibody. The last lanes of blots A, C and D show the elution of MYSM1 upon immunoprecipitation of HDAC1, HDAC2, or MTA2. The last lane of blot B shows the elution of HDAC2 upon immunoprecipitation of MYSM1.

Loss of MYSM1 leads to increased p53 K379 Acetylation

It has previously been shown that there is an interaction between p53 and MYSM1 proteins[54]. Normally, p53 acetylation by pCAF is associated with transcriptional activation[88]. Interestingly, upon HDAC inhibition, p53 has been found to be stabilized and acetylated at lysines 320, 373 and 382[125, 126]. The next step was to determine what kind of effect the loss of MYSM1 has on p53 acetylation and for this, a *Mysm1* shRNA knockdown in Ba/F3 hematopoietic progenitor cells was used in comparison to a firefly luciferase shRNA (shFF) control line. These cells were subjected to irradiation with X-rays at the dose of 3Gy, and lysates were taken at different time points afterwards (0.5hr, 1hr, 2hr, 3hr, 4hr, and 6hr). Total p53 was expressed at low levels in the untreated samples and at higher levels at all time-points following the irradiation (Fig. 12). Specifically, the 1hr time-point showed the highest p53 protein levels relative to all the time-points and the 1 and 2hr time-points also showed slightly greater p53 levels for the shMysm1 cells in comparison to the shFF cells. There was greater K379 acetylation of p53 in the *Mysm1* knockdown cells at the 1hr time point. p53 K320 acetylation was not detected, suggesting that its levels are low in this experimental setting. Overall, the increase in p53K379 acetylation in *Mysm1*-knockdown cells is consistent with the model whereby when MYSM1 is not present, HDACs are not recruited for p53 deacetylation, resulting in increased p53 activation in *Mysm1*-knockdown cells.

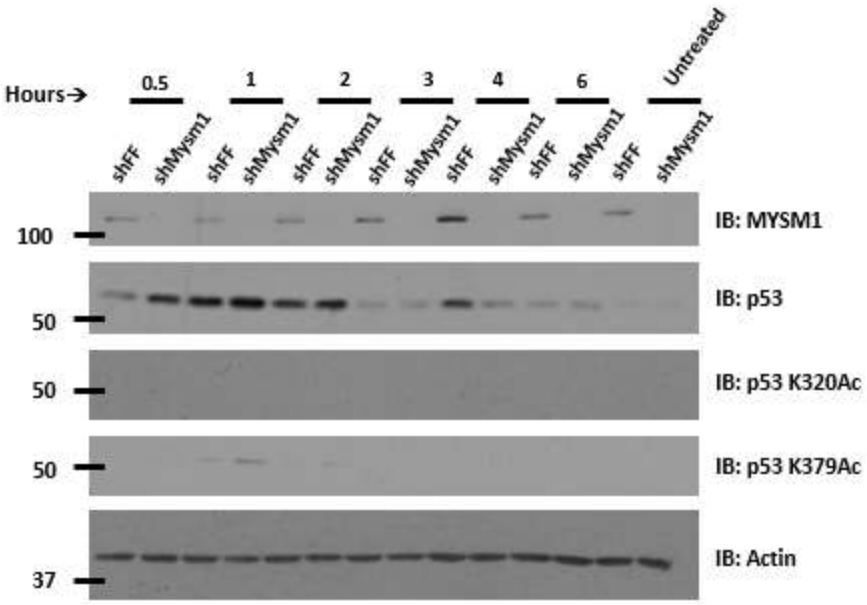

Figure 12: Loss of MYSM1 leads to increased p53 K320 Acetylation.

shFF (control) and shMysm1 knockdown Ba/F3 cells were subjected to 3Gy irradiation and cell lysates were taken at 0.5, 1, 2, 3, 4, and 6 hours. We show that K-379 is acetylated after cell stress and that there is greater acetylation in shMysm1 versus shFF cells. The first lane for each time point is the shFF sample and the second lane is the shMysm1 sample. The result is representative of two independent experiments.

Loss of MYSM1 potentially results in reduced HDAC2 recruitment

Recently, it was shown that MYSM1 localizes to known p53 binding sites at *Cdkn1a* and *Bbc3* promoters[55]. When *Mysm1* expression is knocked down, there is an increase in histone activation markers such as H3K27 acetylation and H3K4 tri-methylation at these sites that correlated with increased gene expression. Our data further indicates that MYSM1 protein interacts with HDACs. Together this leads to the hypothesis that HDACs get recruited with MYSM1 for transcriptional silencing of *Cdkn1a, Bbc3* and potentially other p53-target genes.

To test this, ChIP-qPCR studies were done using Ba/F3 hematopoietic progenitor cells on which a *Mysm1* shRNA knockdown was employed. The shMysm1-knowdown line showed a decrease in *Mysm1* expression both at the transcript and protein levels relative to the firefly luciferase shRNA (shFF) control line (Fig. 13A). The ChIP-qPCR studies were done at steady state (no treatment) and after subjecting cells to ionizing radiation (3Gy, X-rays) to enhance the p53 stress response. The qPCR primers used for these experiments were designed for known p53 binding sites at the *Cdkn1a* and *Bbc3* promoters, discovered through previous ChIP-sequencing data from Kenzelmann Broz D. *et al* (Fig. 13B)[138]. Recruitment of HDAC1 and HDAC2 to known MYSM1 and p53 binding sites was assessed by ChIP-qPCR.

For the sites 841bp and 133bp upstream of the *Bbc3* promoter (-841bp and -133bp), there was a decrease in HDAC2 recruitment when MYSM1 is absent as seen across 3 irradiated biological replicates (Fig. 13C, Table 4A, B). Decrease in HDAC2 recruitment to the *Bbc3* (-841bp) locus was also consistently observed in untreated *Mysm1*-knockdown cells; however this was not the case for the *Bbc3* (-133bp) locus (Fig. 13C, Table 4A, B). For the *Cdkn1a* Isoform 1 TSS site and the Isoform 2 TSS site, ChIP-qPCR showed an overall decreased HDAC2 recruitment in the absence of MYSM1, however it was not reproducible across all biological

replicates (Table 5A, B). The inconsistency can be due to the fact that certain areas of the genome purify better than others, and sonication does not always create breaks at random. Overall, the data provides some support for our hypothesis that when MYSM1 is not present, HDAC2 does not get recruited to certain sites within *Bbc3* and potentially other p53-regulated promoters, which, in turn allows for their transcriptional activation. The data on HDAC1 recruitment to the *Cdkn1a* and *Bbc3* p53-target promoters in the absence of MYSM1 was inconclusive, with different trends seen across multiple biological replicates (data not shown).

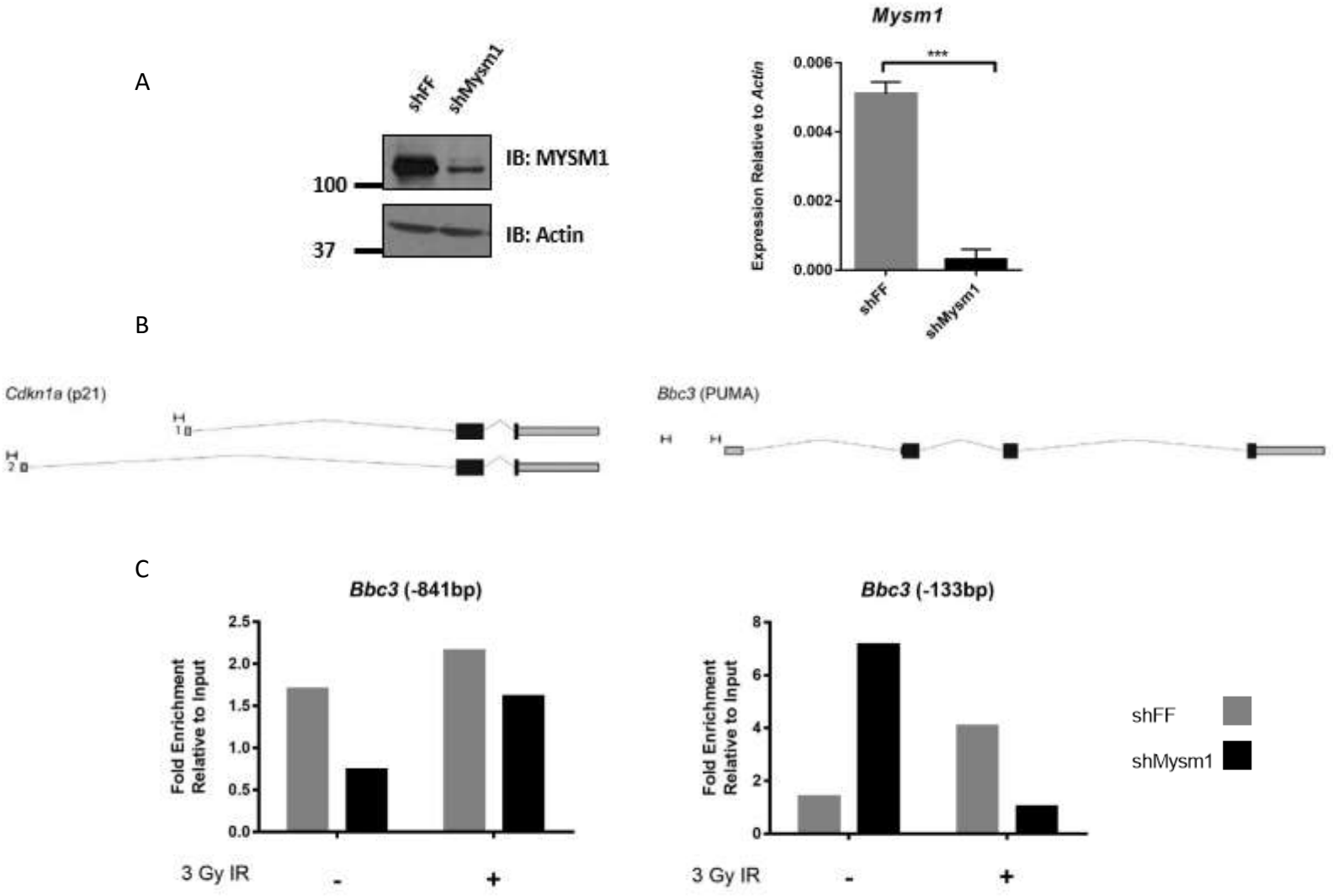

Figure 13: Loss of MYSM1 potentially results in reduced HDAC2 recruitment.

(A) Expression levels of MYSM1 protein and *Mysm1* transcript in shFF and shMysm1 Ba/F3 cells. (B) Genomic structure of *Cdkn1a*/p21 loci and *Bbc3*/PUMA according to the UCSC Mouse mm9 assembly with target primer sites and PCR products indicated (to scale). (C) Enrichment of HDAC2 at the *Bbc3* (-841bp) loci (n=3) and *Bbc3* (-133bp) loci (n=4) in shFF and shMysm1 Ba/F3 cells analyzed by ChIP-qPCR. There is a general decrease in HDAC2 binding at known p53 binding sites within *Bbc3* gene promoter following irradiation in shMysm1 cells as compared to control shFF cells. The data is reproducible across 3-4 biological replicates, with the data from individual replicates presented in Table 4.

Table 4: HDAC2 enrichment at the *Bbc3* promoter measured by ChIP-qPCR.

(A) Enrichment of HDAC2 at the *Bbc3* (-133bp) loci in untreated and irradiated (3Gy) shFF (control) and shMysm1 knockdown cells. (B) Enrichment of HDAC2 at the *Bbc3* (-841bp) loci in untreated and irradiated (3Gy) shFF (control) and shMysm1 knockdown cells. This data is representative of 3-4 biological replicates. The data from replicate #1 was used for figure 13C.

A

Bbc3 (-133bp)	**Replicate #1***	**Replicate #2**	**Replicate #3**	**Replicate #4**
shFF, Untreated	1.405	0.979	0.019	0.969
shMysm1, Untreated	7.146	0.873	0.352	0.634
shFF 3Gy, IR	4.082	0.059	0.392	1.217
shMysm1 3Gy, IR	1.022	0.887	0.230	0.917

B

Bbc3 (-841bp)	**Replicate #1***	**Replicate #2**	**Replicate #3**
shFF, Untreated	1.698	3.739	0.877
shMysm1, Untreated	0.742	1.546	1.412
shFF 3Gy, IR	2.157	1.721	2.252
shMysm1 3Gy, IR	1.611	1.170	1.327

Table 5: HDAC2 enrichment at the *Cdkn1a* promoter measured by ChIP-qPCR.

(A) Enrichment of HDAC2 at *Cdkn1a TSS-1* in untreated and irradiated (3Gy) shFF (control) and shMysm1 knockdown cells. (B) Enrichment of HDAC2 at *Cdkn1a TSS-2* in untreated and irradiated (3Gy) shFF (control) and shMysm1 knockdown cells. This data is representative of 6 biological replicates.

A

Cdkn1a TSS-1	**Replicate #1**	**#2**	**#3**	**#4**	**#5**	**#6**
shFF, Untreated	4.676	5.219	2.195	9.230	0.078	0.824
shMysm1, Untreated	3.458	9.836	1.627	0.963	0.423	0.015
shFF 3Gy, IR	8.863	16.563	15.806	26.308	0.704	0.139
shMysm1 3Gy, IR	10.905	21.701	13.235	10.193	1.705	0.091

B

Cdkn1a TSS-2	**Replicate #1**	**#2**	**#3**	**#4**	**#5**	**#6**
shFF, Untreated	2.638	1.574	1.098	0.502	0.710	1.013
shMysm1, Untreated	0.618	1.235	1.707	0.559	1.095	0.762
shFF 3Gy, IR	3.949	1.800	1.673	1.086	0.630	0.831
shMysm1 3Gy, IR	0.747	3.463	0.007	0.777	2.935	1.417

Loss of MYSM1 results in increased recruitment of K379Ac p53

It has been shown that the acetylation of human p53 at lysine 382 due to the HDAC inhibition by Depsipeptide induces the expression of p21, a downstream regulator of the p53 pathway[129]. Therefore, when HDACs cannot target their sites, acetylation allows for transcriptional activation. Based on this information, ChIP studies were done to determine the effect of *Mysm1* knockdown on the p53 acetylation of the mouse counterpart, lysine 379. Experiments were done using shFF and shMysm1 knockdown Ba/F3 hematopoietic progenitor cells at steady state and after subjecting cells to ionizing radiation (3Gy, X-rays) to enhance the p53 stress response. The primers used were the same as those used for the HDAC1 and HDAC2 ChIP studies (Fig.13B). Recruitment of acetylated p53 to known MYSM1 and p53 binding sites was assessed by ChIP-qPCR. There was a noticeable increase in the recruitment of K379Ac p53 in the absence of MYSM1 at the *Cdkn1a* Isoform 2- TSS site and the *Bbc3* (-133bp) locus across all biological replicates following irradiation (Fig 14, Table 6, 7). The *Cdkn1a* Isoform 2 TSS site and the *Bbc3* (-841bp) locus were also analyzed, however, the results were variable across multiple experiments (data not shown). Overall, these findings provide some support for our hypothesis that when MYSM1 is not present, and in turn HDAC2 is not recruited, p53 can get acetylated and recruited to specific sites at the *Cdkn1a* and *Bbc3* promoters.

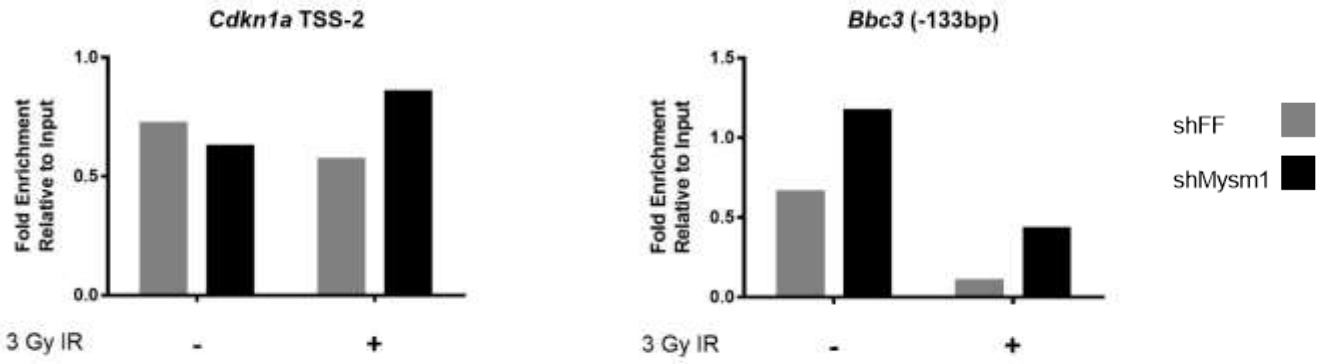

Figure 14: Loss of MYSM1 results in increased recruitment of K379Ac p53.

Enrichment of K379 acetylated p53 at the *Cdkn1a* and *Bbc3* promoters in *Mysm1*-knockdown (shMysm1) and control (shFF) Ba/F3 cells analyzed by ChIP-qPCR. The data is reproducible across 3 biological replicates, with the data from individual replicates presented in Table 6 and 7.

Table 6: p53K379Ac recruitment at the *Cdkn1a* promoter measured by ChIP-qPCR.

Enrichment of p53K379Ac at *Cdkn1a TSS-2* in untreated and irradiated (3Gy) shFF (control) and shMysm1 knockdown cells. This data is representative of 3 biological replicates. The data from replicate #1 was used for figure 14.

Cdkn1a TSS-2	**Replicate #1***	**Replicate #2**	**Replicate #3**
shFF, Untreated	0.721	1.369	1.525
shMysm1, Untreated	0.626	0.812	1.807
shFF 3Gy, IR	0.572	0.947	1.361
shMysm1 3Gy, IR	0.856	1.169	2.096

Table 7: p53K379Ac recruitment at the *Bbc3* promoter measured by ChIP-qPCR.

Enrichment of p53K379Ac at the *Bbc3* (-133bp) locus in untreated and irradiated (3Gy) shFF (control) and shMysm1 knockdown cells. This data is representative of 3 biological replicates. The data from replicate #1 was used for figure 14.

Bbc3 (-133bp)	**Replicate #1***	**Replicate #2**	**Replicate #3**
shFF, Untreated	0.660	1.227	1.174
shMysm1, Untreated	1.1693	0.938	0.745
shFF 3Gy, IR	0.102	0.620	0.642
shMysm1 3Gy, IR	0.426	0.988	0.867

Discussion

p53 is a transcriptional regulator important in cell stress that has been shown to interact with MYSM1[54]. A loss of *Mysm1* in mice results in features of bone marrow failure such as reduced white and red blood cell counts, hematocrit and blood hemoglobin content as well as a severe reduction in the B lymphocytes starting from the early pre-pro B cell stage and defects in T-cell development[54]. Notably, this is associated with activation of p53 in *Mysm1*-deficient HSCs [53]. A *Mysm1*$^{-/-}$*p53*$^{-/-}$ double knockout results in the hematopoietic and phenotypic rescue of the *Mysm1*$^{-/-}$ phenotype[54]. The levels of MPPs, CLPs and CMP hematopoietic progenitor cell populations, and mature hematopoietic cells such as B cells, T cells, and NK cells in the *Mysm1*$^{-/-}$ *p53*$^{-/-}$ mice are similar to their wildtype counterparts[54]. Additionally, the *Mysm1*$^{-/-}$*p53*$^{-/-}$ mice have a restoration of their physical phenotype as their tails are no longer absent, their body length and weight are normal and they have no white belly spots[54]. These findings imply that *Mysm1* antagonizes p53 in mouse development and hematopoiesis. Using Co-IP, our lab has shown that the MYSM1 protein binds p53[54]. Using ChIP, we have shown that when MYSM1 is present, it blocks p53 recruitment and histone modifications that are normally associated with transcriptional activation (histone H3K27Ac), however when MYSM1 is absent, transcription at p53 target promoters, such as *Cdkn1a* (p21) and *Bbc3* (PUMA) can proceed as there is no inhibition[55]. These findings show that MYSM1 is a regulator of HSC activity through p53. The exact mechanism through which this occurs is yet to be understood.

MYSM1 has also been shown to interact with different components of the NuRD complex, importantly HDAC2, in mouse embryonic stem cells, while HDAC2 has been shown to deacetylate p53[91, 121]. In order to gain a better understanding of the mechanism through which

MYSM1 plays a role in the p53 transcriptional network, its interaction with components of the NuRD complex was studied.

We demonstrated that exogenously expressed human MYSM1 protein interacts with HDAC2 in H1299 cells by pulling down the FLAG-tagged HDAC2 or 6x His-tagged MYSM1 (Fig. 11A, B). The His antibody is rabbit polyclonal which means unspecific binding could be a reason as to why there is a strong signal for the IgG mouse sample and not the IgG rabbit sample. We also showed that there is a potential interaction of MYSM1 with HDAC1 and MTA2, which are additional components of the NuRD complex (Fig. 11C, D). The fact that there are two different bands for MYSM1 in the HDAC1 immunoprecipitates indicates that MYSM1 may be post-translationally modified when it interacts with HDAC1.

We attempted to validate the interaction between endogenously expressed MYSM1 and HDAC proteins in Ba/F3 cells, however the interference from Ig heavy chain at approximately 50kDa prevented detention of HDACs in the MYSM1-immunoprecipitates. This technical problem will need to be resolved in future studies. Ba/F3 cells are a pre-pro B cell line, which means they express high amounts of the Ig heavy and light chains. An alternative option would be to use HPC7 cells, which are a hematopoietic precursor cell line, and would express lower amounts of the Ig chains.

HDAC inhibition has shown to lead to an increase in p53 acetylation at lysine 320, 373, and 382[119-121]. For this reason the effect of the loss of MYSM1 on p53 acetylation was studied. If the presence of MYSM1 negatively regulates HDACs, than there should be increased p53 acetylation when MYSM1 is absent. In an irradiation time-course experiment using shFF (control) and *Mysm1*-knockdown cells, at the 1hr time point, there were greater total levels of p53, as well as greater K379 acetylation of p53 in the *Mysm1*-knockdown relative to control cells

(Fig. 12). The p53 levels peaked at the 1hr time point and then slowly tapered down. The fact that there is greater p53 and acetylated p53 at the earlier time points after irradiation-induced DNA damage implies that the cells have started the repair responses, which causes a negative feedback of p53 that lowers its levels at later time points. Also, the lack of MYSM1 causes a greater increase in K379Ac p53 (specifically at the 1 and 2hr time points), supporting our hypothesis that MYSM1 could be a binding partner and positive regulator of HDACs.

Using ChIP studies, we showed that the loss of MYSM1 potentially results in reduced HDAC2 recruitment at the promoters of *Cdkn1a* and *Bbc3* genes that we have previously shown to be transcriptionally co-regulated by MYSM1 and p53 [55]. Importantly, there is a decrease in the recruitment of HDAC2 to the *Bbc3* promoter (-841bp and -133bp) in *Mysm1*-knockdown relative to control cells after 3Gy, X-ray irradiation (Fig. 13C). The consistent decrease in HDAC2 recruitment to the *Bbc3* promoter in comparison to the *Cdkn1a* promoter could be caused by a promoter specific regulation of MYSM1. These findings suggest that MYSM1 and HDAC2 localize to and modify the histones at p53-target promoters. We also showed that the loss of MYSM1 leads to an overall increase in the recruitment of K379Ac p53 at the *Cdkn1a* and *Bbc3* promoters (Fig. 14). For these ChIP studies however, the difference is more evident at the *Cdkn1a* promoter. Nevertheless, the increased recruitment of acetylated p53 in the absence of MYSM1 implies a positive regulatory role of MYSM1 with respect to HDACs.

Overall, the findings in this study suggest that MYSM1 possibly recruits HDAC2 to known p53 binding sites, at least within the *Bbc3* gene promoter and possibly within the promoters of other p53-regulated genes that are also over-expressed in *Mysm1*-knockdown cells. Further investigation is still required to understand the full mechanism of p53 regulation via MYSM1.

Future directions include ruling out possible off-target effects by using additional shRNAs in follow-up experiments. Also, it will be important to test whether the increase in p53 target gene expression (at the transcriptional and protein levels) in *Mysm1* knockdown cells that has been shown in the lab[55] is HDAC dependent. Some supporting evidence can be obtained by applying HDAC inhibitors to wild type Ba/F3 cells to see if an increase in *Cdkn1a*, *Bbc3* and other p53-regulated gene expression can be induced with HDAC-inhibition, mimicking the effects of *Mysm1*-deficiency. This would provide some evidence to show that the effects seen in *Mysm1* knockdown cells, such as increased p53 recruitment and histone H3K27 acetylation at the *Bbc3* and *Cdkn1a* promoters, are due to lack of MYSM1-mediated HDAC-recruitment. An additional step would be to generate HDAC knockdown cells to see the overall effects in p53 transcriptional regulation.

Furthermore, though we have shown that MYSM1 is recruited to known p53 binding regions at p53 target promoters (*Cdkn1a* and *Bbc3*), and that there is potentially less recruitment of HDACs at these same locations when MYSM1 is absent, it is still unknown if this occurs simultaneously, or if the binding of one influences the recruitment and binding of the other. This is an issue since the interdependent binding of different proteins and transcriptions factors and epigenetic modifications are general complicated, and need to be fully understood to suggest a mechanism. In order to address this issue, sequential ChIP assays can be done, which can provide data about the co-occupancy of proteins at p53 target promoters. These assays are also known as "reChIP" since they involve two consecutive ChIPs[139-143]. The first antibody is removed and then a second IP is done, however, a limitation is the DNA recovery at the end, which is very small[144]. Linear DNA amplification can be used to circumvent the problem, but this method is still difficult to use [144].

Biologically, we have shown that the effects of MYSM1 are mediated by p53, so that loss of MYSM1 leads to p53 activation in HSPCs and a p53-mediated hematopoietic failure[54]. MYSM1 is a deubiquitinase with several known substrates, including monoubiquitinated histone H2AK119 and K63-polyubiquitinated TRAF3/6 signaling proteins, however neither of these substrates has any obvious connections to the regulation of p53. Furthermore, we did not observe changes in H2AK119ub levels at p53-regulated promoters *Bbc3* and *Cdkn1a* in *Mysm1*-knockdown cells (JI Belle, unpublished data). For this reason, it is also important to understand if the catalytic activity of MYSM1 is important for its activity as a p53 inhibitor in hematopoiesis. To address this, mutant retroviral vectors encoding MYSM1 with reduced or no catalytic activity were developed by targeting the JAMM domain. The mutant design was based on the study of another DUB, AMSH-LP, which has the same set of the conserved residues for Zn^{2+} coordination as MYSM1[145]. One mutant (E588A) targets the water-activating residue, while the other (S657, D660N) targets the intermediate-stabilizing and Zn^{2+} coordinating residues respectively. *Mysm1* knockout fetal liver HSCs (CD45.2) were transduced with *Mysm1*^{WT}, *Mysm1*^{E588}, *Mysm1*$^{S567A,\ D660N}$ constructs or an empty vector. These cells were injected into wild type irradiated mice (CD45.1) to determine if there is a reconstitution of the hematopoietic system. Future students in the lab will perform bleed analysis of the mice at 8 and 16 weeks, followed by a final analysis at 20 weeks. It is expected that the *Mysm1*^{WT} construct will lead to a rescue of HSC functions and hence a reconstitution of the hematopoietic system of the recipients by the injected donor *Mysm1*^{WT}-HSC, while the empty vector will not. If the *Mysm1* mutant constructs do not allow for reconstitution, than that means that the catalytic activity of MYSM1 is necessary for its activity, but if they do, than it means that the catalytic activity of MYSM1 is

not necessary. This information would allow us to understand if the MYSM1 catalytic activity is important for its activity as a p53 inhibitor in hematopoiesis.

Once there is a better understanding of the mechanism behind the regulation of p53 by MYSM1, activation of p53 by repressing MYSM1 could be a feasible strategy for the treatment of hematological cancers since p53 mutations are less common in hematological malignancies than in solid tumors[146-151].

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