CONTROL OF THE TUMOR SUPPRESSOR p53 BY REGULATING MDM2 ACTIVITY AND STABILITY

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This dissertation is dedicated to the memory of H. T. Chandrasena.

He was an explorer, a husband, and father of three that was taken too early by the hidden enemy we strive to fight as researchers, cancer.

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ABSTRACT

CONTROL OF THE TUMOR SUPPRESSOR p53 BY REGULATING MDM2 ACTIVITY AND STABILITY

Ruchira S. Ranaweera

Xiaolu Yang

p53 is a tumor suppressor that is widely mutated or deleted in cancer cells. Mdm2, an E3 ubiquitin ligase, is the master regulator of p53. It targets p53 for proteasomal degradation, restraining the potent activity of p53 and enabling cell survival and proliferation. There are complex regulatory mechanisms balancing the activity and stability of Mdm2 in a cell. Mdm2 has an extremely short half-life in the unstressed cell and its regulation is not well understood. Like most E3 ligases, Mdm2 can autoubiquitinate. Previously, the sole function of autoubiquitination was thought to be to signal Mdm2 degradation. Here I show that autoubiquitination of Mdm2 is an activating event. Mdm2 that has been conjugated with polyubiquitin chains exhibits substantially enhanced activity to polyubiquitinate p53. Mechanistically, autoubiquitination of Mdm2 facilitates the recruitment of the E2 ubiquitin-conjugating enzymes through non-covalent interactions between the ubiquitin chains on Mdm2 and the ubiquitin-binding domain on E2s. These results suggest a model in which polyubiquitin chains on an E3 increase the local concentration of E2 enzymes and permit the processivity of substrate ubiquitination. These results support the notion that autocatalysis may be a prevalent mode for turning on the activity of latent enzymes.

Mdm2 is a short-lived protein because it is ubiquitinated and targeted for proteasomal degradation. In the unstressed cell, a complex containing the adaptor protein Daxx and HAUSP help stabilize Mdm2 protein. Through a collaborative effort, we discover BRG1, an ATPase component of the human SWI/SNF chromatin remodeling complexes, as a novel component of the Mdm2-Daxx-HAUSP complex. We find that BRG1 interacts with and enhances the assembly of this complex. Interestingly, I find that BRG1 is essential for maintaining Mdm2 levels independently of its ATPase activity. Moreover, BRG1 controls cell proliferation, senescence, and transformation through the stabilization of Mdm2. These results define BRG1 as an essential component of the Mdm2-Daxx-HAUSP complex and reveal an activity of BRG1, beyond chromatin remodeling, that is required for cell survival. Altogether, these results provide novel insights into the intricate mechanisms regulating the activity and stability of the oncoprotein Mdm2, enabling its negative control of the tumor suppressor p53.

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CHAPTER 1 : Introduction

1.1. p53: A Brief History

Discovery of p53

p53 is possibly the most well studied gene and protein in cancer biology, with over 50,000 PubMed-listed publications to date. Widely accepted as the "guardian of the genome", p53 has risen to fame in the last 15 years as the most often mutated or deleted tumor suppressor in human cancer. More than 50% of human tumors have deletions or mutations of p53 while those with wild-type p53 may contain alterations of regulators and effectors of p53.

Identification and Mischaracterization

Discovered simultaneously by several independent groups in 1979, p53 was first identified as a large T-antigen interacting protein with an approximate molecular mass of 53 kDa in the extracts of SV40 virus transformed cell lines (DeLeo et al. 1979; Kress et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979). This newly discovered protein was present at high levels mainly in transformed cell lines but not in non-transformed cells. Moreover, it interacted with other viral tumor antigens like E1B-55Kd encoded by another small DNA tumor virus. In 1984, several groups unknowingly cloned mutated mouse and human p53 cDNA using mRNA from transformed cell lines that were abundant in p53 protein. The fact that p53 interacted with viral tumor antigens and was highly abundant in transformed cells lent further support to its

categorization as a cellular oncogene. Scientists used the newly cloned p53 cDNA to demonstrate its ability to immortalize cells, cooperate with oncogenes such as HRAS, and even increase the *in vivo* tumorigenic properties of p53-null cells (Arnold J Levine and Oren 2009). Consequently p53 was acknowledged as an oncogene, with its significance and mechanism of action to be discovered years later.

Mutations and mouse models establish p53 as a tumor suppressor

Nevertheless, there were clues all along that p53 might be a tumor suppressor rather than an oncogene. Observations were made in mouse and human cancer cell lines that the p53 gene was interrupted by retroviral insertion or extensively rearranged, preventing production of functional p53 protein (Arnold J Levine and Oren 2009). Alleles of mutated p53 were discovered in patient samples of colorectal carcinoma. The implications of these findings were not clear at the time. Questions were raised about p53's transforming role when Cathy Finlay and Phil Hinds in Levine's lab cloned yet another p53 cDNA, showing for the first time that overexpression of a "wild-type" p53 clone suppresses rather than promotes transformation of rodent cells (Hinds, Finlay, and Levine 1989). All of these findings led to a paradigm shift in the field, ultimately ending with the recognition of p53 as a *bona fide* tumor suppressor (A J Levine 1989).

The first transgenic mice carrying a mutant p53 gene were created in 1989. Almost 20% of the mutant p53 transgenics had a high incidence of tumor development, providing direct evidence that mutant p53 has oncogenic properties *in vivo* (Lavigueur et al. 1989). Around the same time, germline mutations of the human p53 gene *TP53*

were detected in families with Li-Fraumeni syndrome, characterized by early-onset cancers of various types (Malkin et al. 1990; Srivastava et al. 1990). Even though *p53*-null mice were viable with no obvious developmental defects, *p53* deletion predisposed the mice to lymphomas and sarcomas, and all *p53*-null mice died by 8 months of age due to excessive tumor burden (Donehower et al. 1992). Mice heterozygous for *p53* retained cancer predisposition albeit with a much delayed onset and varied frequencies of tumor types (Harvey et al. 1993). These p53 mouse models and the germline mutations in human patients cemented the classification of p53 as a tumor suppressor. Although p53 was not essential for embryonic development, it was crucial to suppress tumorigenesis in mice and humans. The specific functions and mechanisms of action for p53 were revealed through many additional studies.

1.2. Tumor suppressor functions of p53

Stress signals activate p53

In an unstressed cell, p53 is strictly maintained at a very low level by continuous ubiquitination and subsequent degradation by the 26S proteasome. Various cellular stresses such as nutrient deprivation, oncogene activation, DNA damage, and hypoxia lead to stabilization and rapid nuclear accumulation of p53 (**Figure 1.1**). Activated p53 is a tetrameric sequence-specific transcription factor capable of inducing transcription of a large network of genes. The majority of these p53 target genes mediate outcomes such as apoptosis, cell-cycle arrest, senescence, DNA repair, and differentiation. The

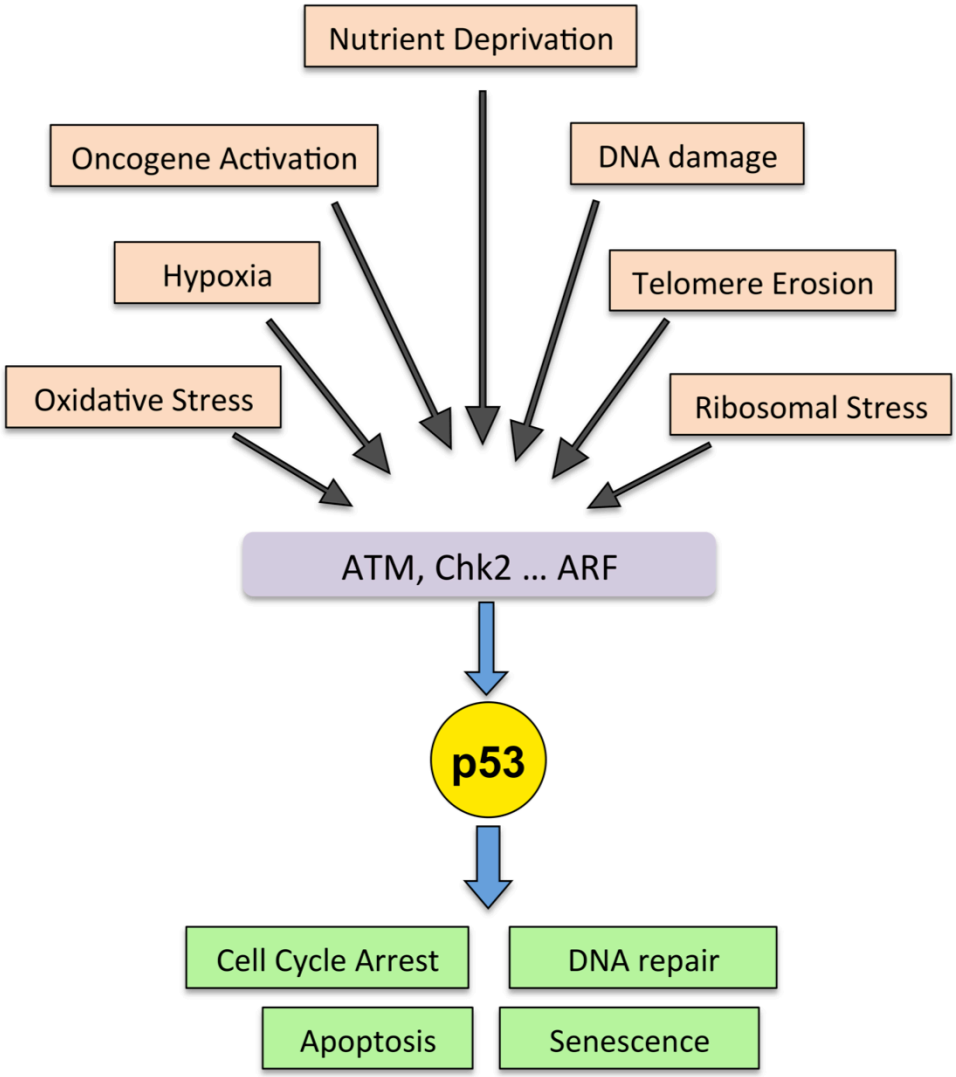

Figure 1.1. The p53 response pathway.
Different cellular stresses can activate p53 via mediators, leading to the transcriptional induction of various genes resulting in diverse cellular outcomes.

major cell cycle arrest target of p53 is a cyclin-dependent kinase (CDK) inhibitor, p21, encoded by *Cdkn1a*. Induction of p21 inhibits G1 to S transition of the cell cycle by disrupting the activation of Cyclin E- and Cyclin A-CDK complexes (Vousden and Lu 2002). This execution of reversible cell cycle inhibition demonstrates one major tumor suppressive function of p53 as it gives the cell the time it needs to repair its damaged DNA before the next cell cycle.

In other cases, p53 activates pro-apoptotic target genes such as Noxa, PUMA and Bax, which turn on the intrinsic apoptotic pathway (Vousden and Lu 2002). Alternatively, p53 has also been implicated in the induction of replicative-senescence of damaged cells. Although the exact mechanisms are unclear, evidence suggests that p53 may induce senescence via p21 induction (Vousden and Prives 2009). Altogether, the p53-mediated activation of cell cycle arrest, apoptosis and senescence are crucial in maintaining genomic integrity, earning p53 its title as the "guardian of the genome".

Post-translational Modification of p53

Although the exact mechanisms of p53 activation under various stresses are unclear, key upstream regulators mediate the stabilization and activation of p53 by targeting p53 for post-translational modification. The mediators may change depending on the context of stress signaling. In response to DNA damage induced by ionizing radiation or ultraviolet (UV)-light radiation, the upstream kinases ATM and Chk1 phosphorylate p53 on specific serine residues, increasing its sequence-specific DNA binding (Bode and Dong 2004). In addition to phosphorylation, p53 is regulated by acetylation (CBP/p300, PCAF, SIR2), methylation, sumoylation, ubiquitination and

many other post-translational modifications, which affect its stability and transactivation. The single most important mode of p53 regulation is its degradation via ubiquitination. The principal negative regulator of p53 is the RING E3 ubiquitin ligase Mdm2 (Mouse double minute 2).

1.3. Mdm2: an oncogenic E3 ligase for p53

The Mdm2 oncogene negatively regulates p53

Mdm2 was originally identified as one of three genes that was amplified in the transformed mouse cell line 3T3-DM, leading to greater than 50-fold overexpression of Mdm2 protein (Fakharzadeh, Trusko, and George 1991). The moniker 'mouse double minute' arose from the localization of the *Mdm* genes to small, acentromeric extrachromosomal nuclear bodies, called double minutes (Cahilly-Snyder et al. 1987). Soon, it was discovered that overexpression of Mdm2 was responsible for cellular transformation, demonstrating its role as an oncogene (Finlay 1993).

Not long after its discovery, Mdm2 rose to fame when it was found to interact with and inhibit the transactivation of p53 (Momand et al. 1992). *Mdm2* gene amplification was also seen in over 30% of human sarcomas that retained wild-type p53 (Oliner et al. 1992). This led to the hypothesis that overexpression of Mdm2 may be a mechanism that cancer cells use to inactivate wild-type p53 in the process of tumor development. Studies have shown that amplifications of *Mdm2* in approximately 7% of human tumors account for at least one mechanism of Mdm2 overexpression. A more recent publication has uncovered that a naturally occurring polymorphism (SNP309)

within the *Mdm2* promoter leads to an increase in *Mdm2* mRNA and protein in the human population (Arva et al. 2005).

The best genetic evidence for the importance of Mdm2 in controlling p53 is the early embryonic lethality by day 5.5 of *Mdm2*-null mice due to extensive uncontrolled apoptosis. Remarkably, simultaneous genetic deletion of *p53* rescues the *Mdm2*-null phenotype, suggesting that the death of *Mdm2*-null mice is due to spontaneous p53 activation during development (Jones et al. 1995; Montes de Oca Luna, Wagner, and Lozano 1995). It also indicates that the main function of Mdm2, at least during embryonic development, is to regulate p53.

The Mdm2-p53 regulatory loop

In the unstressed cell, Mdm2 associates with and targets p53 for degradation by the 26S proteasome (Fuchs et al. 1998). DNA damage disrupts the Mdm2-p53 association, with subsequent degradation of Mdm2 and the accumulation of p53 (**Figure 1.2**). Interestingly, the *mdm2* gene itself is a direct transcriptional target of p53 in response to DNA damage, thereby forming a negative feedback loop to regulate p53 stability and activation (Barak et al. 1993; Perry et al. 1993; Wu et al. 1993). The newly translated Mdm2 negatively regulates p53 in two ways: Mdm2 binds to the p53 transactivation domain to directly inhibit its ability to activate transcription and Mdm2 uses its E3 ubiquitin ligase activity to polyubiquitinate p53 and target it for subsequent

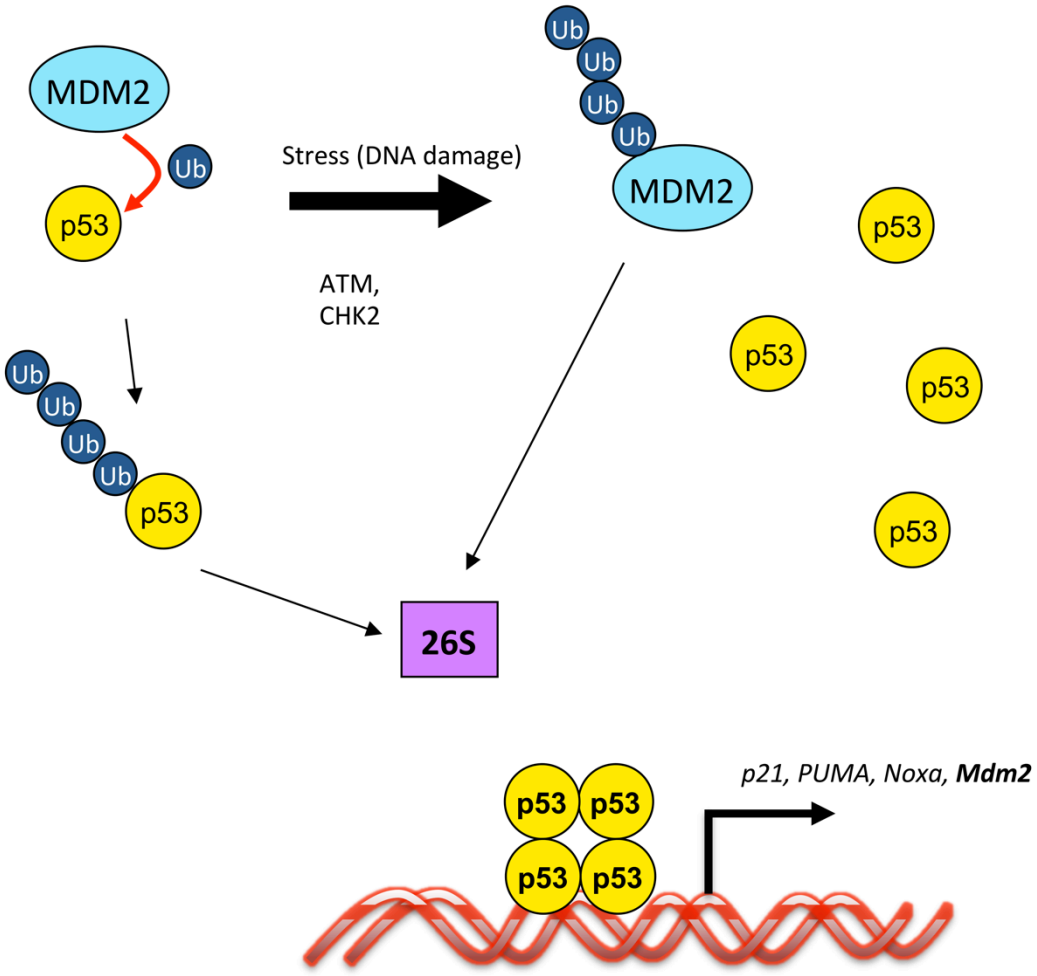

Figure 1.2. p53 ubiquitination by Mdm2.
In the unstressed cell, Mdm2 polyubiquitinates p53 targeting it for degradation by the 26S proteasome. Upon DNA damage, Mdm2 is ubiquitinated and degraded, allowing p53 accumulation. Tetrameric p53 functions as a transcription factor to mediate transcriptional activation of various genes, including Mdm2. Newly synthesized Mdm2 attenuates the p53 response and brings the cell back to an unstressed state.

proteasomal degradation, thereby rapidly reducing the p53 protein level to bring the cell back to an unstressed state.

MdmX, an Mdm2 homolog and p53 regulator

Members of the RING finger ubiquitin ligase family can function as monomers, dimers, or multi-subunit complexes. Dimerization is generally mediated through their RING domains and they can form homodimers (RNF4, Mdm2, Siah, Traf2, cIAP) as well as heterodimers (BRCA1/Bard1, Ring1b/Bmi1). Mdm2 forms a heterodimeric complex with MdmX (also known as Mdm4), a RING domain containing Mdm2 homolog. Initially discovered in a mouse cDNA screen for p53-interacting partners (Shvarts et al. 1996), the human ortholog, MDMX, was identified later (Shvarts et al. 1997). MdmX does share many functional characteristics of Mdm2. For example, MdmX can also directly inhibit the ability of p53 to activate transcription (Marine, Dyer, and Jochemsen 2007). *MDMX* is overexpressed or alternatively transcribed in almost 30% of tumor cell lines tested that also retained wild-type p53. Furthermore, 19% of breast carcinomas and a significant percentage of other tumor types analyzed revealed *MDMX* overexpression (Danovi et al. 2004; Marine, Dyer, and Jochemsen 2007). *MdmX*-null mutations in mice are early embryonic lethal, due to loss of cell proliferation and uncontrollable p53 activation, highlighting its role as a p53 regulator *in vivo*. Concomitant deletion of *p53* in mice rescues the developmental defects caused by *MdmX* loss (Finch et al. 2002; Migliorini et al. 2002; Parant et al. 2001). Therefore, Mdm2 and MdmX are non-redundant critical regulators of p53 function *in vivo*.

Unlike Mdm2, *MdmX* is not a transcriptional target of p53 and its mRNA levels do not change during the DNA damage response. In response to DNA damage, Mdm2 targets MdmX for ubiquitination and subsequent degradation. Although it possesses a RING domain nearly identical to that of Mdm2, MdmX itself has no intrinsic ubiquitin ligase activity (Wade, Wang, and Wahl 2010).

Mdm2 and MdmX structure and function

Mdm2 and MdmX are structurally similar, each at 491 and 490 amino acids in length respectively (**Figure 1.3**). The two proteins are highly conserved at the N-terminus, which contains the p53-binding domain. Mdm2-p53 and MdmX-p53 interactions also require the same residues within p53. The second zinc-finger of Mdm2 and MdmX is also conserved. The central acidic domain, with no significant similarity between the two proteins, has no attributed function as yet. The C-terminal RING domain of the 'rare' C2H2C4 type is highly conserved, and its structural integrity is crucial to mediate Mdm2-MdmX heterodimerization (Linke et al. 2008). Mutation of the conserved cysteine residue, 464 and 462 in Mdm2 and MdmX respectively, causes a loss of function and disrupts their dimerization. Mdm2, unlike MdmX, has a nuclear localization signal (NLS) and a nuclear export signal (NES) (Wade, Wang, and Wahl 2010).

There is growing evidence for functional interplay between Mdm2 and MdmX. Many groups are now discovering that an Mdm2-MdmX heterodimer may be the predominant form *in vivo.* In fact, data suggests that interaction of MdmX with Mdm2 can safeguard Mdm2 from degradation (Stad et al. 2001; Tanimura et al. 1999). MdmX

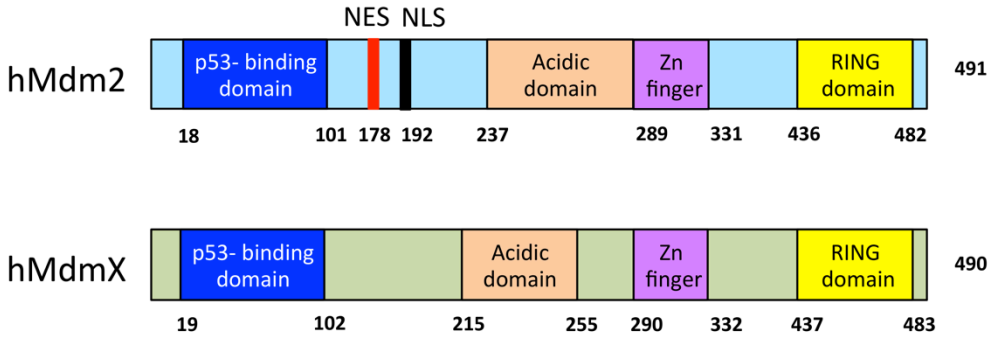

Figure 1.3. Mdm2 and MdmX domain architecture.
A schematic outlining similarities and differences in structure between human Mdm2 and its homolog MdmX.

overexpression can even counteract the negative effects of the tumor suppressor ARF on Mdm2 by becoming an alternate SUMOylation substrate for ARF (Ghosh, Weghorst, and Berberich 2005). The current consensus in the field is that MdmX and Mdm2 cooperate to efficiently ubiquitinate p53 *in vivo* (Linares et al. 2003; Poyurovsky et al. 2007; Uldrijan, Pannekoek, and Vousden 2007). The Mdm2-MdmX heterodimer is a more potent and abundant E3 ligase complex than monomeric Mdm2 (Kawai et al. 2007).

Mdm2's E3 activity is not required for its degradation

Mdm2 is a member of the large family of RING domain-containing E3 ubiquitin ligases (discussed in Chapter 1.2). Well known as the major E3 ligase for p53, Mdm2 also demonstrates activity toward itself. Intriguingly, autoubiquitination is a general feature of Mdm2 and other RING E3 ligases (Fang et al. 2000). Autoubiquitination was long thought to be solely responsible for Mdm2 degradation. How the opposing activities of *cis* and *trans* ubiquitination are regulated remains elusive.

A recent mouse knock-in model of Mdm2 contributed an interesting piece of information (Itahana et al. 2007). Homozygous mice expressing Mdm2, with a single-residue substitution (C462A) eliminating E3 function, died early in development before E7.5, but could be rescued by p53 deletion. Interestingly, the mutant $Mdm2^{C462A}$ demonstrated a half-life indistinguishable from that of wild-type Mdm2. The study also examined stability of the mutant Mdm2 at physiologically relevant levels compared with ectopically expressed Mdm2. Only ectopically expressed $Mdm2^{C462A}$ demonstrated a longer half-life than wild-type Mdm2. Altogether, this study changed how we perceive

Mdm2 autoubiquitination. Contrary to the previous model where self-activity of Mdm2 targeted it for proteasomal degradation, Mdm2 E3 function is not required for its degradation. This raises the possibility that Mdm2 autoubiquitination may have an unexplored function.

1.4. Ubiquitination Pathway and Mechanisms

Ubiquitination

Covalent conjugation to ubiquitin, a highly conserved 76-amino acid peptide, is a major post-translational modification that regulates the stability, function, and localization of proteins. Notably, attachment of a ubiquitin chain can serve as a signal for the removal of proteins through the ubiquitin proteasome system, a major pathway for intracellular protein degradation. Ubiquitination targets the vast majority of cellular proteins and plays major roles in cell cycle progression, differentiation, DNA damage responses and tumor suppression.

Ubiquitination takes place due to the sequential actions of three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (**Figure 1.4**). The E1 cleaves and activates the C-terminal glycine of ubiquitin in an ATP-dependent manner, forming a thioester bond with ubiquitin. Activated ubiquitin is then transferred to the active-site cysteine of an E2 enzyme to produce an E2 thioesterified with ubiquitin (E2~Ub). The specificity and efficiency of ubiquitination are largely determined by the E3, which binds to both the E2~Ub and a substrate protein, and stimulates the transfer of ubiquitin from E2~Ub to a lysine

residue on the substrate. A protein can be conjugated at one or multiple sites with a single ubiquitin (monoubiquitination) or a polyubiquitin chain (Cecile M Pickart 2004).

Formation of polyubiquitin chains linked to each other via their Lys48 residues marks a protein for degradation by the 26S proteasome (Chau et al. 1989). Alternatively, polyubiquitination linked via Lys63 can lead to non-proteolytic consequences in many cases (Scialpi et al. 2008; C. Wang et al. 2001). Effective polyubiquitination of substrates is critical for protein degradation and various non-degradative processes including signal transduction. An E3 must consecutively add four or more ubiquitin in a single encounter with a substrate, in case the substrate dissociates prematurely and other enzymes remove the ubiquitin. However, the mechanism for the processive assembly of polyubiquitin chains remains poorly understood.

Mechanisms of RING domain E3 ligases

There are two major families of E3s in eukaryotes, distinguished by having either a HECT (homologous to the E6-AP carboxyl terminus) or RING (really interesting new gene) domain. While there are approximately 30 HECT domain ligases, there are over 600 RING ubiquitin ligases in the mammalian cell. Upon interaction with an E2~Ub thioester, a HECT E3 forms a ubiquitin-thioester intermediate using a catalytic cysteine within the HECT domain, prior to ubiquitin transfer onto a specific substrate (Metzger, Hristova, and Weissman 2012). The RING domain binds to E2 enzymes and promotes direct transfer of ubiquitin to a substrate

Certain RING domain proteins such as cullin-RING ligases (CRLs), function as a part of multiprotein complexes where substrate recognition is mediated by a different subunit of the complex (Deshaies and Joazeiro 2009). Because the protein subunits of CRLs are interchangeable, it adds a great deal of diversity to their substrate specificity. However, most RING E3 ligases are relatively large proteins that can function as single-molecule E3s because they contain the RING domain and the substrate interaction domain on the same peptide (Ex: Mdm2, BRCA1, Ring1b).

Unlike the HECT domain E3s, RING E3s do not form an intermediate thioester with ubiquitin prior to substrate ubiquitination. The RING domain provides an interaction pocket for the E2~Ub that catalyzes discharge of its ubiquitin while a different domain of the protein interacts with the specific substrate. The specific pairing of E2-E3 enzymes can determine the type and length of ubiquitination output (Ye and Rape 2009). For RING E3s, a major rate-limiting step in the formation of a polyubiquitin chain is the recruitment of E2~Ub to the E3 (Deshaies and Joazeiro 2009). Because an E2 interacts with both the E1 and the E3 RING domain through overlapping regions, it needs to dissociate from the RING domain to be re-thioesterified with ubiquitin (**Figure 1.4**). Previous studies have shown that the cullin-based RING ligases circumvent this rate-limiting step, in part, through rapid association and disassociation between an E2 and the E3 RING domain (Kleiger et al. 2009). A separate E2 binding site on the E3 subunit mediates this dynamic interaction. Yet the mechanism by which single subunit RING E3s achieve processive ubiquitination is not known. A notable trait of RING-containing E3s is their autoubiquitination (de Bie and Ciechanover 2011). At least one study has demonstrated the ability of autoubiquitination to enhance the

activity of a RING-domain E3 toward its substrate (Mallery, Vandenberg, and Hiom 2002). Despite being commonly regarded as an experimental measure of *in vitro* enzymatic activity, the function of E3 autoubiquitination is not well defined.

There are some RING domain proteins that do not possess intrinsic E3 activity. MdmX, Bard1, and Bmi1 all posses RING domains but do not demonstrate any E3 activity on their own (Metzger, Hristova, and Weissman 2012). Each of these three inactive RING proteins interacts with active RING E3 ligases (Mdm2, BRCA1, Ring1b, respectively) to form heterodimeric complexes. In each case, heterodimerization greatly stimulates the E3 activity of the active RING E3 ligase. Although many groups have studied these heterodimeric complexes, it is still unclear how dimerization enhances E3 activity of these ubiquitin ligases.

E2s and Ubiquitin Binding Domains

E2 conjugating enzymes contain a topologically conserved core domain of ~150 residues (C M Pickart 2001). The function of this core domain is required to coordinate ubiquitin transfer onto a substrate. It must recognize the E1, transport covalently attached ubiquitin, and coordinate with E3 ligases to facilitate ubiquitin transfer onto a substrate. Some E2s contain accessory domains that confer added functionality. These additional elements often belong to a structurally diverse group of domains collectively named ubiquitin-binding domains (UBDs) (Hicke, Schubert, and Hill 2005).

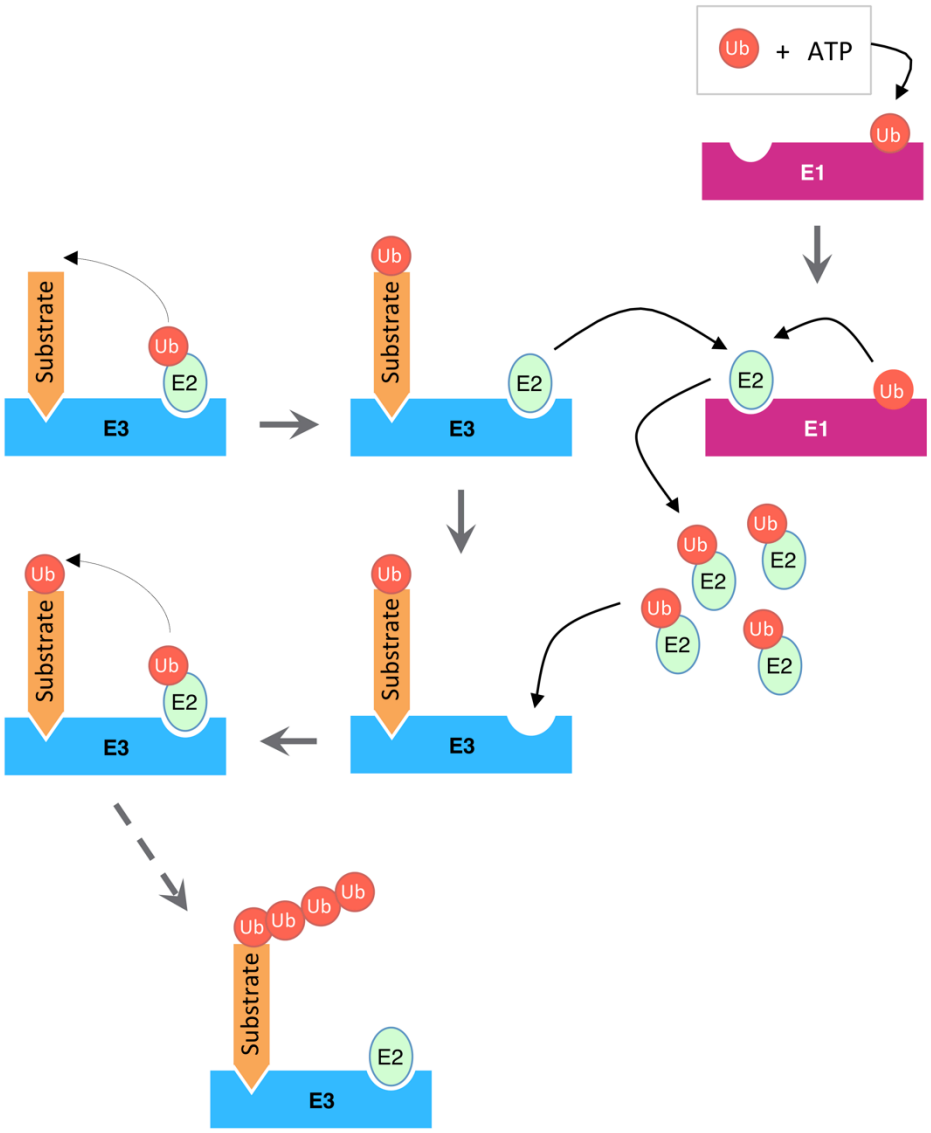

Figure 1.4. Ubiquitination.
Reaction scheme depicting a RING-domain E3 ligase going through multiple rounds of E2~Ub recruitment in order to facilitate polyubiquitination of a specific substrate

All UBDs are able to recognize the same surface on the ubiquitin molecule and form a non-covalent complex (**Figure 1.5**). Although the UBD-ubiquitin interactions are relatively weak (Kd = 5-500mM), structural integrity of the domain is essential for biological function of an E2.

Members of the UbcH5 family of E2s are the physiological ubiquitin conjugating enzymes for Mdm2-mediated p53 ubiquitination (Saville et al. 2004). UbcH5c, a member of this E2 family, was also recently found to contain a UBD with the ability to form non-covalent complexes with ubiquitin (Brzovic et al. 2006). The study further demonstrated the importance of the E2 UBD in facilitating polyubiquitination of BRCA1, a single-molecule RING E3 ligase. It is still unclear how the non-covalent interaction between an E2 and ubiquitin contributes to the reaction mechanism of ubiquitination.

Deubiquitinating enzymes

Ubiquitination is a reversible modification, which can be removed by a superfamily of isopeptidases termed deubiquitinases (DUBs) (Komander, Clague, and Urbé 2009). They can be subdivided into five families: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Josephins and JAB1/ MPN/MOV34 metalloenzymes (JAMMs; also known as MPN+ and hereafter referred to as JAMM/MPN+). Less than a hundred DUBs have been identified to date, suggesting a low degree of selectivity. However, DUBs are also subject to multiple layers of regulation that fine-tune both their activity and specificity. The contribution of

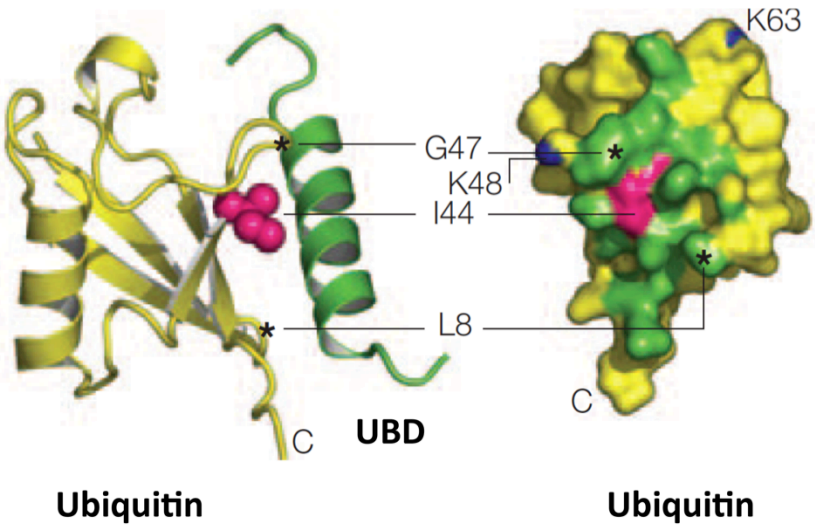

Figure 1.5. Structures of an E2 ubiquitin-binding domain and ubiquitin. Shown is a ubiquitin:UBD complex structure determined by using X-ray crystallography and NMR spectroscopy. The left panel shows a ribbon diagram of ubiquitin (yellow), and the UBD (green). Ile44 of ubiquitin is shown in a red, spacefill representation. The right panel shows ubiquitin with the UBD contact surfaces colored green, although the Ile44 side chain is again shown in red and is part of the contact surface. The amino groups of Lys48 and Lys63, which are two of the key sites for polyubiquitin chain conjugation, are coloured blue. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Molecular Cell Biology] (Hicke, Schubert, and Hill 2005), copyright (2005).

deubiquitination mediated by USP7 to the p53-mdm2 axis is discussed in the following section.

1.5. Protein complexes regulating Mdm2 stability

Deubiquitination by the Daxx-HAUSP complex

Conjugated ubiquitin can be removed through the action of a class of isopeptidases termed deubiquitinases (DUBs). Removal of ubiquitin from a substrate can allow for fine-tuning or reversal of the modification. Previous members of our laboratory found that the deubiquitination of Mdm2 is mediated through interactions with the adaptor protein Daxx (death domain-associated protein) and the deubiquitinase HAUSP (Herpes-associated ubiquitin specific protease, also known as USP7) (Tang et al. 2006) (**Figure 1.6**). Downregulation of Daxx decreased Mdm2 levels whereas Daxx overexpression increased Mdm2 levels, suggesting that Daxx is required for Mdm2 stability. Daxx simultaneously interacts with HAUSP and Mdm2 to prevent the proteasomal degradation of Mdm2 as well as to enhance Mdm2-mediated ubiquitination of p53. Treatment of cells with etoposide, a DNA damage-inducing agent, disrupted the interaction between Mdm2 and the Daxx-HAUSP complex. Phosphorylation of specific serine residues on Daxx and Mdm2 by the kinase ATM, a first responder in the DNA damage response, leads to the disassembly of the Mdm2-Daxx-HAUSP complex (Tang et al. 2013). Without HAUSP-mediated deubiquitination, Mdm2 is highly ubiquitinated and degraded in response to DNA damage, allowing for activation of the p53 response. The tumor suppressor RASSF1A was recently found to

promote Mdm2 ubiquitination and degradation by interacting with Mdm2 and Daxx, preventing the stabilizing effects of the Daxx-HAUSP complex (Song et al. 2008). There may be intricate mechanisms in place to regulate the Mdm2-Daxx-HAUSP complex, and in turn p53 activation.

BRG1: a SWI/SNF family ATPase and a tumor suppressor

To investigate the mechanism of the Daxx-Mdm2-Hausp complex in regulating p53, we set out to identify new components of this complex and identified BRG1 (Brahma-related gene 1) as a candidate protein. Mutation or silencing of BRG1 is associated with various human cancers but its mechanisms of function are unclear (Wilson and Roberts 2011). BRG1 is an ATP-dependent chromatin remodeler which uses energy derived from ATP hydrolysis to slide, insert or evict histones and actively alter the nucleosomal structure (Johnson, Adkins, and Georgel 2005).

The packaging of genomic DNA into chromatin is viewed as a major obstacle to the process of transcription. The nature of packaged chromatin plays a fundamental role in the regulation of gene expression. Chromatin remodeling proteins are able to manipulate the chromatin structure and facilitate recruitment of co-regulators essential for transcription. Several chromatin-remodeling complexes have been discovered and classified based on the identity of their central catalytic subunits: SWI/SNF, Ino80, ISWI, and NuRD. Components of the SWI/SNF complex (SWItch Sucrose NonFermentable) are highly conserved in eukaryotes ranging from yeast to humans.

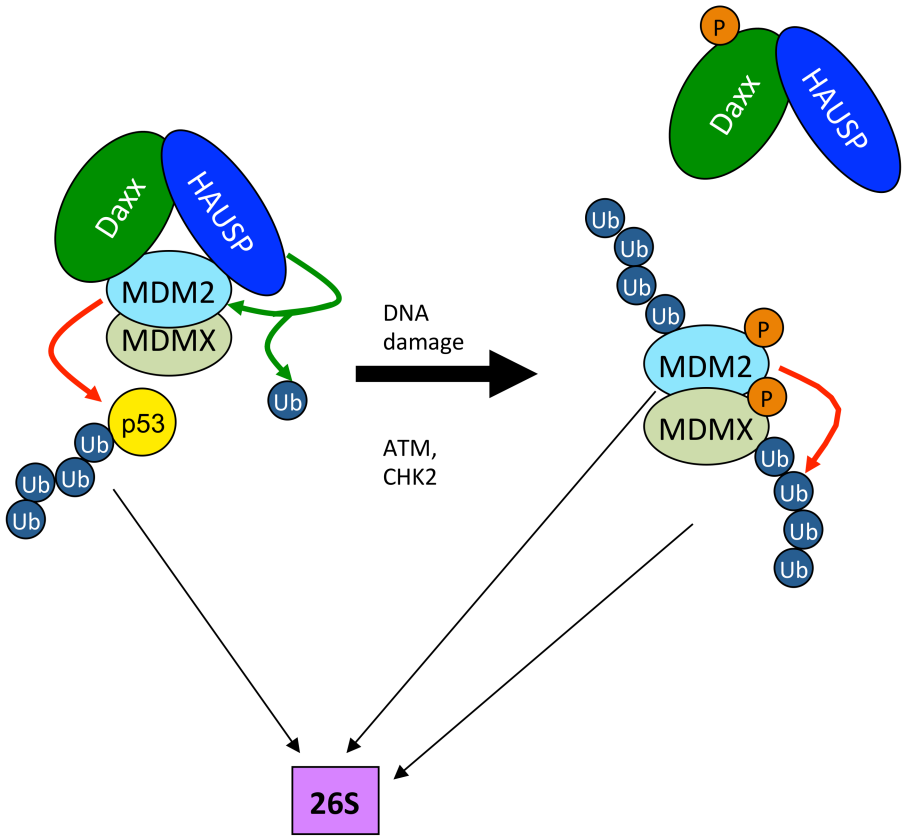

Figure 1.6. Regulation of Mdm2 stability.
In the unstressed cell, Mdm2 heterodimerizes with MdmX. Mdm2 stability is maintained by the Daxx-HAUSP complex through deubiquitination. Mdm2-MdmX dimer targets p53 for degradation by the 26S proteasome. Upon DNA damage, phosphorylation events disrupt the complex. Mdm2 ubiquitinates MdmX. Mdm2 itself is also ubiquitinated and degraded, allowing p53 accumulation.

Human SWI/SNF contains the mutually exclusive BRG1 (Brahma-related gene 1) or BRM (Brahma) proteins as their central ATPase subunit. The BRG1 and BRM proteins are paralogs that display similar biochemical activities *in vitro* and share a high degree (74%) of sequence identity (Khavari et al. 1993; Phelan et al. 1999; Randazzo et al. 1994). The two proteins play distinct but overlapping roles in proliferation, differentiation and other cellular processes using unknown mechanisms of specificity (S. Bultman et al. 2000; Kadam and Emerson 2003; Reyes et al. 1998).

BRG1 Structure and Function

BRG1 is composed of multiple domains, including an evolutionarily conserved catalytic ATPase domain, a C-terminal bromodomain, and an AT-hook motif. Mutation of the highly conserved lysine 783 to arginine in the ATP binding pocket of BRG1 (K798 in yeast SWI2) abolishes BRG1 ATPase activity (Khavari et al. 1993). The BRG1 bromodomain may be responsible for the recognition of acetylated lysines within histone H3 and H4 (Chandrasekaran and Thompson 2007; Shen et al. 2007). Histone modifications such as acetylation may serve to recruit bromodomain-containing complexes to specific target promoters.

The less characterized N-terminal region contains QLQ, HSA and BRK domains. The glutamine-leucine-glutamine (QLQ) domains are often implicated in protein-protein interactions and/or might help determine the structure of BRG1. The function of the HSA domain is unknown but they are found in helicases and other eukaryotic DNA binding proteins. BRK domains, also with no attributed function, are associated with transcription (Trotter and Archer 2008). Altogether, BRG1 is composed of

multiple domains, many of which may contribute to potential protein interaction modules. They may be used to recruit BRG1 to genomic targets via recognition of modified histones or other chromatin bound proteins.

Within a cell, BRG1 is found as an enzymatic subunit of various multi-protein complexes involved in transcriptional regulation and DNA replication, repair, and recombination. In the SWI/SNF complex, BRG1 (or BRM), associates with approximately 10-12 highly conserved core BAFs (BRG1-associated factors), which are heterogeneous in different SWI/SNF complexes. Although BRG1 alone can perform nucleosome remodeling *in vitro*, addition of the core BAF subunits enhances chromatin-remodeling activity to optimal levels (Phelan et al. 1999).

A multitude of BRG1 interacting partners have been discovered over the years, using various methods. Interestingly, BRG1 interacts with a diverse group of nuclear proteins such as nuclear receptors, transcriptional machinery components, chromatin-modifying enzymes, tumor suppressors, and other factors important for genomic stability and maintenance. BRG1 plays roles in DNA repair through interaction with BRCA1 (Bochar et al. 2000) and muscle differentiation via Mef2D (Ohkawa, Marfella, and Imbalzano 2006). BRG1 also interacts with the tumor suppressor Rb (retinoblastoma) and functions in Rb-mediated G1 arrest (Dunaief et al. 1994; D. N. Reisman et al. 2002; Strobeck et al. 2002).

Studies have also demonstrated BRG1 association with p53. Results from one study found that BRG1 and hSNF5, another SWI/SNF component, are necessary for activation of p53-mediated transcription (Lee et al. 2002). More recent work suggests BRG1 may be a critical negative regulator of p53 by interacting with CBP, promoting

polyubiquitination of p53 (Naidu et al. 2009). It is still unclear what role BRG1 plays in regulating p53 and its tumor suppressor functions.

Functional Role for BRG1 in cancer development

Many subunits of the SWI/SNF complex may be involved in cancer development. The BAF47 subunit, a core BRG1-associated factor, was recently discovered as a bona fide tumor suppressor (Wilson and Roberts 2011). Evidence is surfacing for perturbations in the SWI/SNF ATPases, BRG1 and BRM, in cellular transformation. BRG1 and BRM are mutually exclusive ATPases with overlapping but distinct cellular functions (W. Wang et al. 1996). BRG1 tends to be more abundantly expressed in proliferating cells whereas BRM is expressed in differentiated cells. Expression of BRG1 and BRM was uniformly lost in 30-40% of lung cancer cell lines (Decristofaro et al. 2001; D. N. Reisman et al. 2002). BRG1 is also located in an area of the genome that displays loss of heterozygosity in human cancers (Gunduz et al. 2005; Medina et al. 2008), a classic hallmark of proteins that function as tumor suppressors. BRG1 exhibited loss of heterozygosity in 23% of small-cell lung cancer cell lines and 77% of non-small-cell lung cancer lines (Girard et al. 2000). Loss of BRG1 in cancer seems to occur simultaneously with mutations in other oncogenes and tumor suppressors, including p53 (D. Reisman, Glaros, and Thompson 2009). Mouse models have contributed further evidence. Deletion of both BRG1 alleles is embryonically lethal (S. Bultman et al. 2000; Sumi-Ichinose et al. 1997). Knock out of a single allele of BRG1, however, results in 10% of the mice spontaneously developing mammary tumors within one year, suggesting BRG1 haplo-insufficiency leads to tumor formation (S. J.

Bultman et al. 2008; S. Bultman et al. 2000). Interestingly, conditional monoallelic BRG1 knockout in non-transformed lung epithelial cells potentiates tumor development while biallelic knockout induces apoptosis (Glaros et al. 2008). Altogether, BRG1 plays a critical role in cancer development but the mechanisms are unclear.

CHAPTER 2 : Autoubiquitination Regulates Mdm2 Substrate Ubiquitin Ligase Activity

Covalent conjugation to ubiquitin is a major post-translational modification that regulates protein stability, function, and localization. Ubiquitination takes place due to sequential actions of three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). The specificity and efficiency of ubiquitination are largely determined by the E3, which binds to both an E2 thioesterified with ubiquitin (E2~Ub) and a substrate protein, and stimulates the transfer of ubiquitin from E2~Ub to the substrate. The vast majority of the hundreds of known ubiquitin E3s contain a RING (really interesting new gene) domain. Some RING proteins contain only the RING domain and function in multi-subunit E3 complexes (e.g. Cullin-RING ligases or CRLs), but most RING proteins are relatively large proteins with multiple domains and can function as single-molecule E3s.

A protein can be conjugated at one or multiple sites with a single ubiquitin or a poly-ubiquitin chain. Formation of poly-ubiquitin chains is critical for protein degradation and various non-degradative processes including signal transduction. However, the mechanism for the processive assembly of ubiquitin chains remains poorly understood. For RING E3s, a major rate-limiting step for the formation of a poly-ubiquitin chain is the recruitment of E2~Ub to the E3. Because an E2 interacts with both E1 and the E3 RING domain through overlapping regions, it needs to dissociate from the RING domain to be re-thioesterified with ubiquitin. Previous studies have shown that a cullin-RING ligase circumvents this rate-limiting step in part through rapid

association and disassociation between an E2 and the E3 RING domain. Yet the mechanism by which relatively large RING E3s achieve processive ubiquitination is not known. A notable trait of RING-containing E3s is their auto-ubiquitination. Despite being commonly regarded as a mechanism of autocatalytic degradation, the function of E3 auto-modification is not well defined.

The multi-domain RING domain protein Mdm2 is the principal ubiquitin ligase of the tumor suppressor p53. p53 becomes activated in response to cellular stresses such as DNA damage, nutrient deprivation, and oncogene activation. The activation of p53 leads to potent anti-proliferative outcomes ranging from cell cycle arrest to senescence and apoptosis, making the control of p53 levels a central issue in mammalian cells. In unstressed cells, p53 is a short-lived protein largely due to Mdm2-mediated ubiquitination and proteasomal degradation. Mdm2 is also a short-lived protein that undergoes autoubiquitination. Although this was previously thought to cause Mdm2 degradation, subsequent studies have shown that autoubiquitination of Mdm2 is not responsible for Mdm2 degradation *in vivo*. Here I seek to address the function of Mdm2 autoubiquitination. I show that autoubiquitination of Mdm2 can enhance its substrate ubiquitination activity. I also find that autoubiquitination of Mdm2 leads to strong recruitment of E2 conjugating enzymes, overcoming the rate-limiting step of E2 recruitment and increasing the processivity of substrate ubiquitination.

2.1. Autoubiquitination Enhances Substrate Ubiquitin Ligase activities of Mdm2 and the Mdm2-MdmX heterodimer

To examine how Mdm2 autoubiquitination may affect its ability to covalently modify p53, we used an *in vitro* system where autoubiquitination could be readily separated from the subsequent p53 ubiquitination (**Figure 2.1**). Glutathione *S*-transferase (GST)-tagged Mdm2 expressed and purified from mammalian cell extract was immobilized on beads (**Figure 2.1**, top left). GST-mdm2 was incubated with E1, E2, and ubiquitin, in the presence or absence of ATP to permit or prevent Mdm2 autoubiquitination (**Figure 2.2**, A). Afterwards, the ubiquitination reaction components were washed away, and the unmodified and auto-modified Mdm2 were used to conjugate Flag-tagged p53 with ubiquitin in the presence of fresh reaction components. Notably, compared to the unmodified Mdm2, auto-modified Mdm2 exhibited a noticeably stronger ability to ubiquitinate p53 (**Figure 2.2**, A). To exclude any GST tag-specific effects, we performed a similar experiment using hemagglutinin (HA)-tagged Mdm2. Auto-modified HA-Mdm2 also showed a strongly enhanced ability to ubiquitinate p53 (**Figure 2.2**, B). The difference in p53 ubiquitination was more dramatic at the earlier time of points for p53 ubiquitination. It is important to note that the ratio of p53:mdm2 in the ubiquitination reaction contributed to this effect on p53 ubiquitination. The ratio of 1:3, p53 to Mdm2, was determined empirically and consistently demonstrated the activating effects of Mdm2 autoubiquitination.

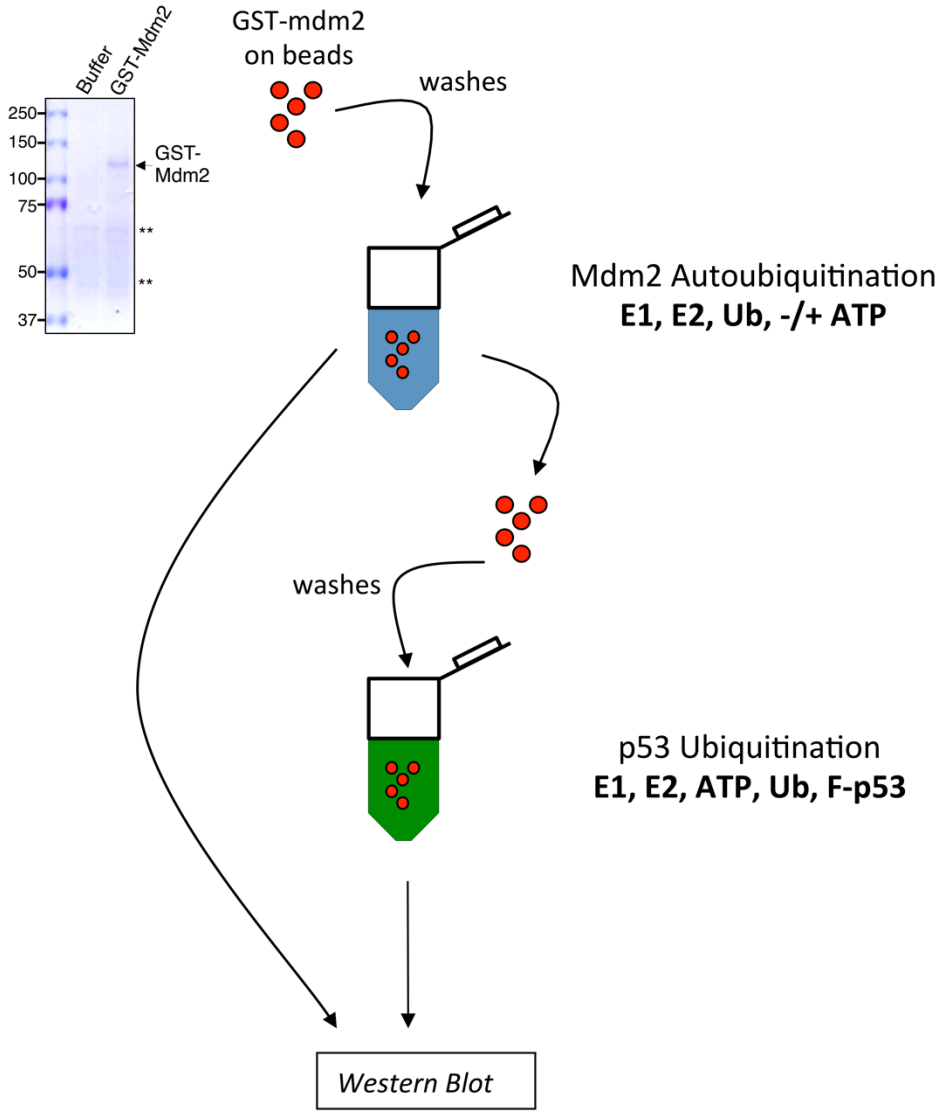

Figure 2.1. *In vitro* Ubiquitination Assay.
Schematic diagram of two-step *in vitro* ubiquitination reaction setup. Coomassie stained SDS-PAGE gel showing purity of bead-immobilized GST-mdm2 (top left). **Non-specific bands.

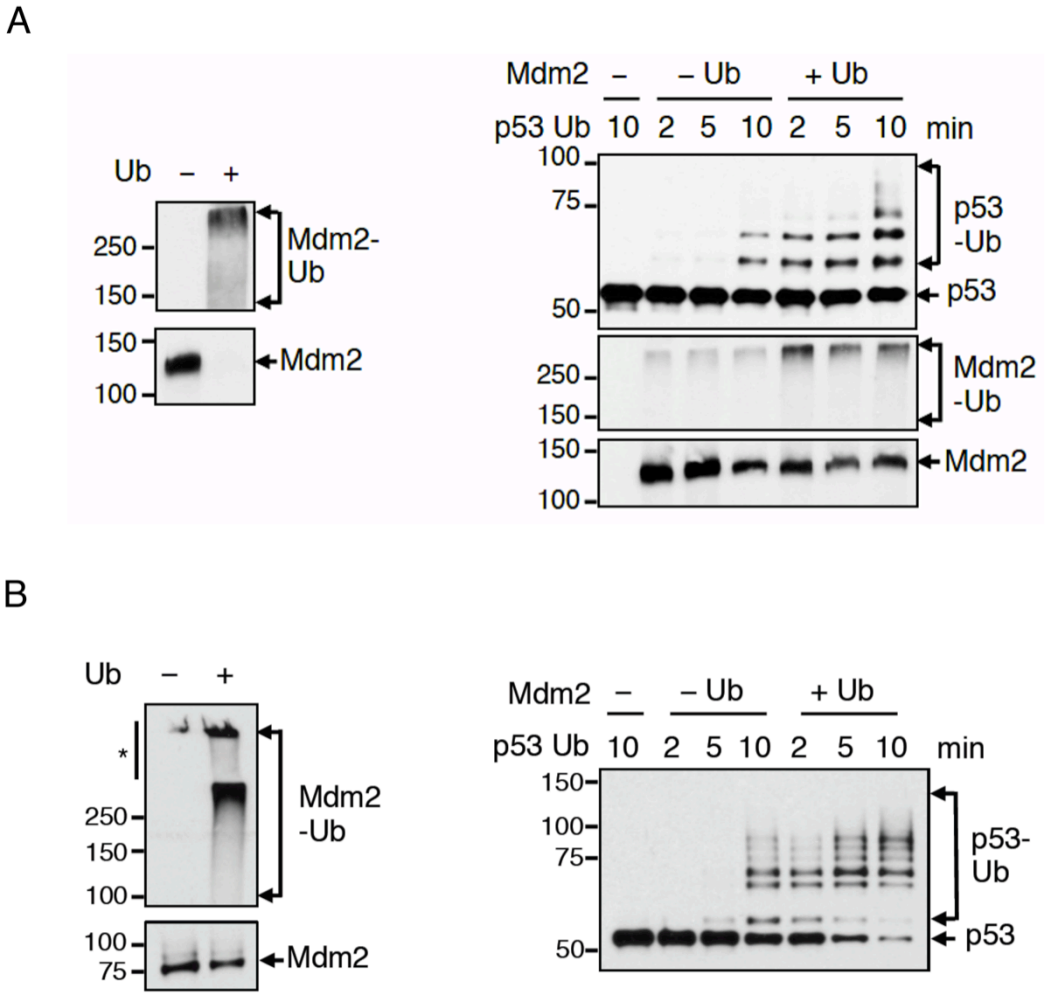

Figure 2.2. Autoubiquitination of GST-mdm2 or HA-mdm2 enhances E3 activity.
Left: Western blot depicting unmodified (-) or autoubiquitinated (+) GST-Mdm2 (*A*) or HA-Mdm2 (*B*) immunoblotted with anti-Mdm2 (bottom left) and anti-ubiquitin (top left). Right: p53 ubiquitination was performed in the presence of no Mdm2 (-), unmodified Mdm2 (- Ub), or autoubiquitinated Mdm2 (+ Ub) and western blot was probed with anti-p53 antibody.

In vivo Mdm2 is present predominantly as a heterodimer with MdmX, which possesses minimal E3 activity of its own, but stimulates the E3 activity of Mdm2 (Linares et al. 2003; Poyurovsky et al. 2007; Uldrijan, Pannekoek, and Vousden 2007). To generate the Mdm2:MdmX complex, we co-expressed GST-tagged Mdm2 and Flag-tagged MdmX in cells and performed sequential pull-downs with anti-Flag antibody-conjugated beads and glutathione beads. Upon auto-ubiquitination, the purified Mdm2:MdmX complex showed markedly enhanced E3 activity towards p53 (**Figure 2.3**). These results suggest that auto-ubiquitination enhances the E3 activity of both Mdm2 and the Mdm2:MdmX complex.

2.2. Auto-ubiquitination of Mdm2 promotes p53 poly-ubiquitination

A poly-ubiquitin chain linked through the Lys48 residue on ubiquitin is the canonical signal for proteasomal degradation (Chau et al. 1989). We examined whether p53 poly-ubiquitination was enhanced by Mdm2 auto-ubiquitination. When auto-modified Mdm2 was used, poly-ubiquitinated p53 species, which were indicated by their reactivity to a poly-ubiquitin-specific antibody as well as by their extremely high molecular weights, appeared rapidly (within two min). In contrast, when unmodified Mdm2 was used, poly-ubiquitinated p53 species appeared relatively slowly (in ~20 min) (**Figure 2.4**). Auto-modified Mdm2 also exhibited an enhanced ability to conjugate p53 with Lys48-only ubiquitin, in which all Lys residues except for Lys48 were mutated to Arg residues (**Figure 2.5**, lanes 1-7). These results suggest that auto-ubiquitination of Mdm2 enhances its ability to conjugate p53 with Lys48-linked poly-ubiquitin chains.

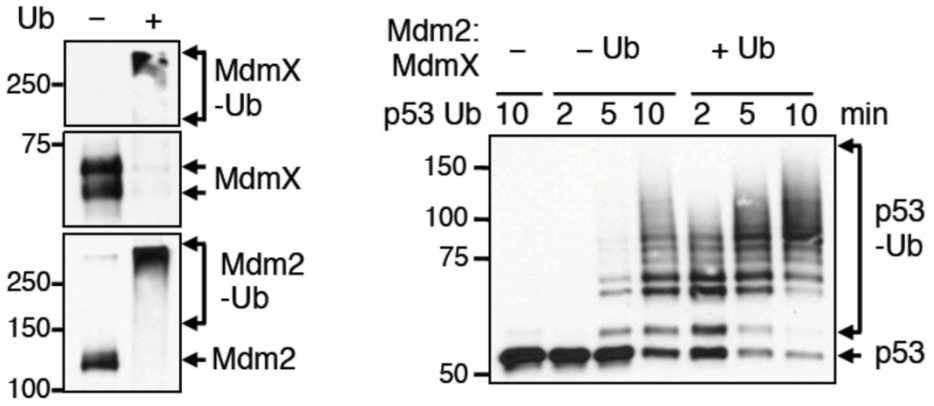

Figure 2.3. Autoubiquitination stimulates activity of the Mdm2-MdmX heterodimeric complex.
Mdm2:MdmX complex that was co-purified from 293T cells was unmodified (-) or autoubiquitinated (+) and analyzed with anti-MdmX (top left and middle left) or anti-Mdm2 (bottom left) antibodies. Ubiquitination of p53 by previously unmodified (- Ub) or auto-modified (+ Ub) Mdm2:MdmX complex was detected with anti-p53 antibody (right).

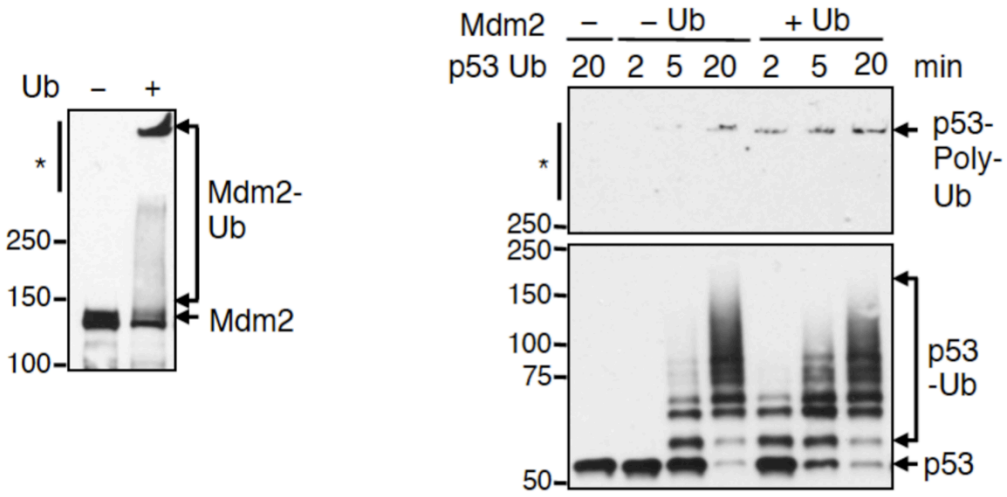

Figure 2.4. Polyubiquitination of p53 is enhanced by autoubiquitinated Mdm2. GST-Mdm2 autoubiquitination was probed with anti-Mdm2 (left) and p53 ubiquitination equivalent to 5% input for immunoprecipitation (bottom right) was analyzed with anti-p53 antibody. A portion of the p53 ubiquitination reaction was used to immunoprecipitate p53 and analyzed with a poly-ubiquitin specific antibody (FK1) for p53 poly-ubiquitination (top right). *: Stacking gel.

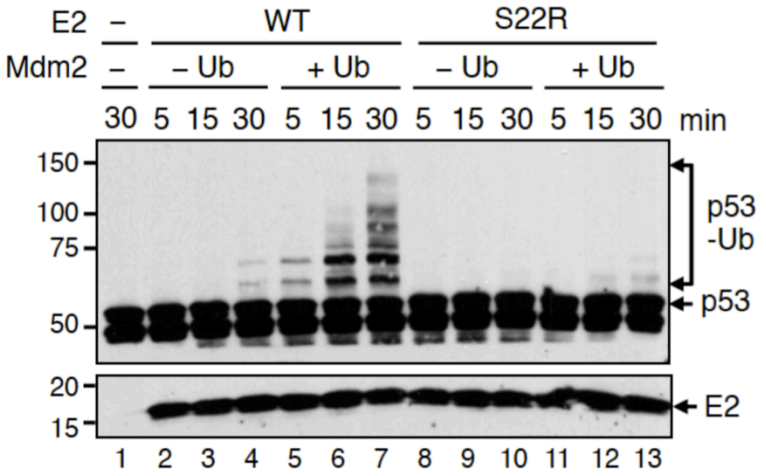

Figure 2.5. Autoubiquitination promotes Mdm2-mediated p53 polyubiquitination and Lysine-48-linked ubiquitination.
GST-Mdm2 that was unmodified or autoubiquitinated with wild-type ubiquitin was used to ubiquitinate p53 using Lys48-only ubiquitin in the presence of no E2, WT UbcH5c, or S22R UbcH5c (in which Ser22 of UbcH5c was mutated to Arg). Antibodies for Western blot were anti-p53 (top) and anti-UbcH5 (bottom).

To examine whether the extent of Mdm2 auto-ubiquitination influences its substrate E3 activity, we performed a time course experiment for Mdm2 auto-ubiquitination. The ability of GST-Mdm2 to ubiquitinate p53 initially increased in relation to more auto-ubiquitination, but later declined with higher levels of auto-ubiquitination (**Figure 2.6**, A). A similar result was observed using HA-Mdm2 (**Figure 2.6**, B). However, regardless of the extent of auto-ubiquitination, auto-modified Mdm2 was consistently more active than unmodified Mdm2 at ubiquitinating p53. To determine whether the increase in Mdm2's substrate E3 activity is due to poly-ubiquitination, we used methylated ubiquitin, which permits only mono-ubiquitination at one or multiple sites (19). The substrate E3 activity of mono-ubiquitinated Mdm2 (Me) was comparable to that of unmodified Mdm2 (**Figure 2.7**), indicating that mono-ubiquitination did not enhance Mdm2-mediated p53 ubiquitination.

2.4. Autoubiquitination does not alter substrate binding

Next we investigated the mechanisms by which auto-ubiquitination of Mdm2 stimulates the substrate E3 activity of Mdm2. A possible explanation is that auto-ubiquitination might enhance the ability of Mdm2 to interact with p53. However, an *in vitro* pull-down assay showed that unmodified and auto-modified Mdm2 were comparable in their binding to p53 (**Figure 2.8**, A).

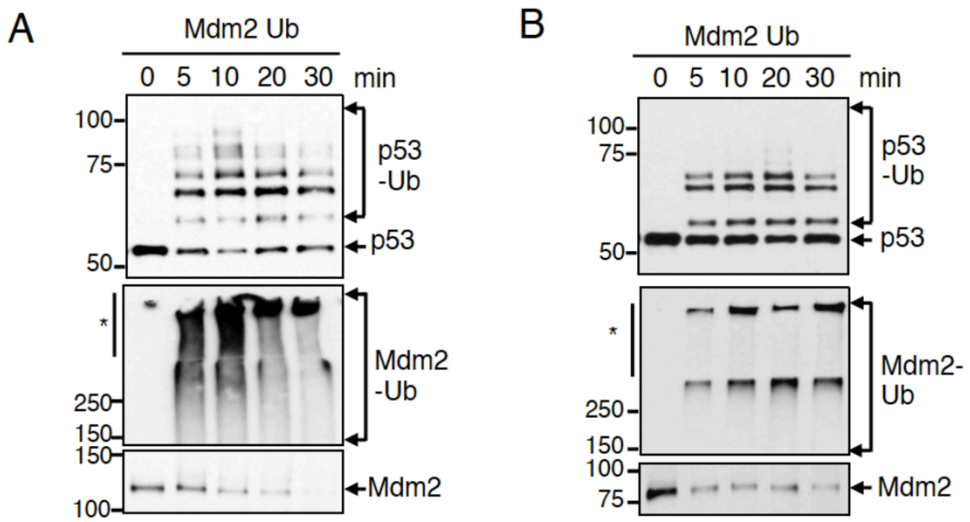

Figure 2.6. The extent of poly-ubiquitination regulates Mdm2 E3 activity. Ubiquitination of p53 with GST-Mdm2 (*A*) and HA-Mdm2 (*B*) that has been auto-ubiquitinated for the indicated times. Ubiquitination of p53 was detected with anti-p53 antibody (top), and Mdm2 autoubiquitination was analyzed with anti-Mdm2 (bottom) and anti-ubiquitin (middle) antibodies.

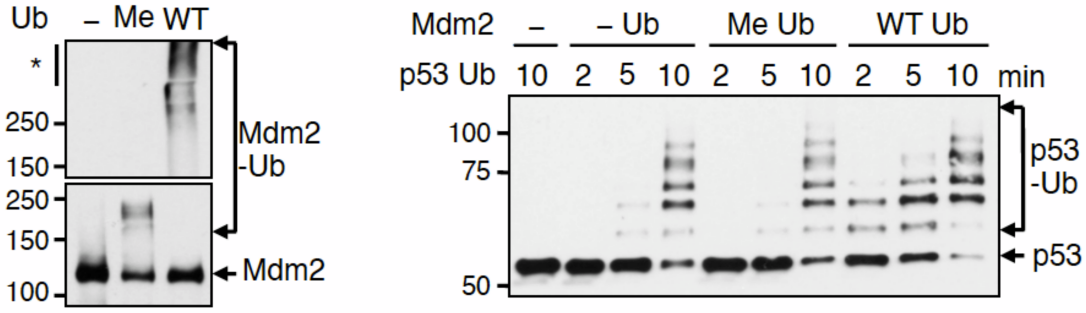

Figure 2.7. Monoubiquitination of Mdm2 does not enhance E3 activity. Ubiquitination of p53 in the presence of no Mdm2 (-), unmodified Mdm2 (- Ub), mono-ubiquitinated Mdm2 (Me Ub), or poly-ubiquitinated Mdm2 (WT Ub). Mdm2 autoubiquitination was probed with anti-Mdm2 (bottom left) and anti-ubiquitin (top left). Ubiquitinated p53 was detected with anti-p53 antibody (right).

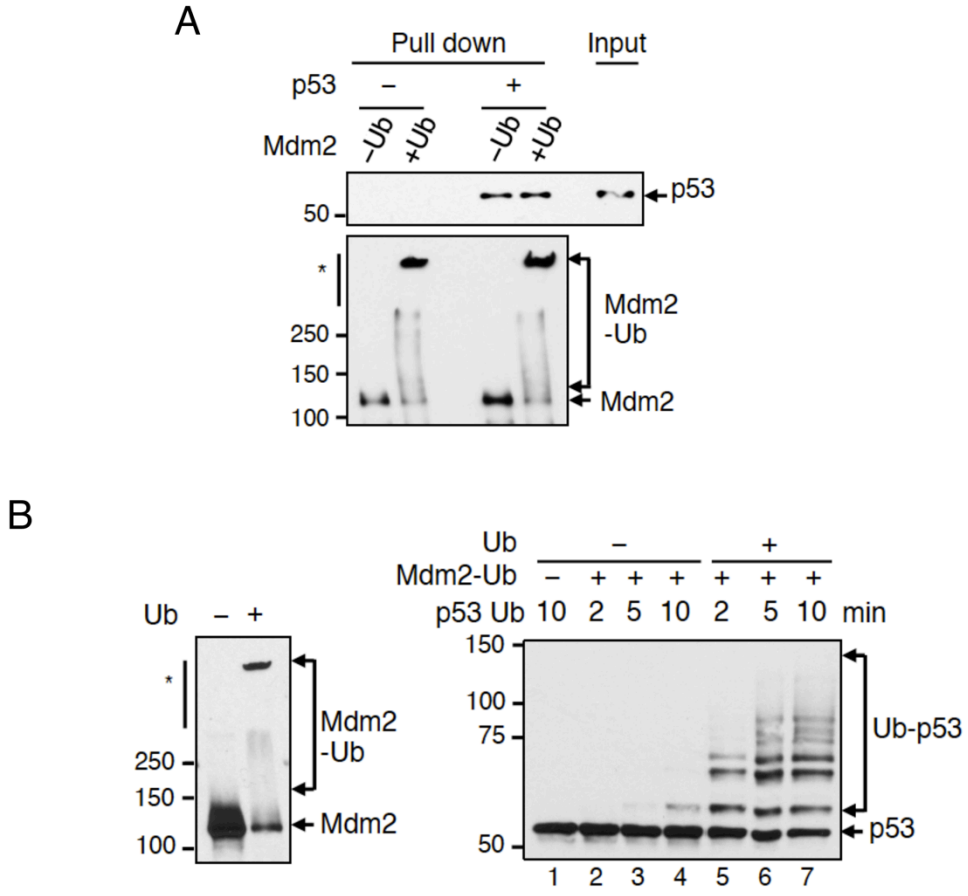

Figure 2.8. Autoubiquitination of Mdm2 does not change affinity for p53 or promote direct ubiquitin transfer.
(*A*) Immobilized GST-Mdm2 with or without auto-ubiquitination was incubated alone or with p53. The bound proteins were analyzed by Western blot with anti-p53 (top) and anti-Mdm2 (bottom) antibodies. The p53 input shown is equivalent to 2.5% of total p53. (*B*) Immobilized GST-Mdm2 was auto-ubiquitinated (left) and used for p53 ubiquitination without (–) or with ubiquitin (+) in the reaction (right).

Additionally, we considered the possibility that Mdm2 may transfer ubiquitin chains assembled on itself to p53. A previous study showed that the RING domain ubiquitin ligase gp78, which is involved in degradation of misfolded endoplasmic reticulum proteins, could transfer to the substrate protein a poly-ubiquitin chain that is attached via a thioester bond to the active site Cys residue of the E2 Ube2g2 (20). For a poly-ubiquitin chain attached via an isopeptide bond to a Lys residue on an E3, such a transfer mechanism has not been reported. To distinguish between *de novo* chain synthesis and ubiquitin transfer, we incubated auto-ubiquitinated GST-Mdm2 with p53 and ubiquitination components but omitted ubiquitin from the reaction. p53 was ubiquitinated in the absence of fresh ubiquitin, but the amount of ubiquitination was very small and accounted for only a minute fraction of total p53 ubiquitination mediated by auto-modified Mdm2 (**Figure 2.8**, B). This result suggests that auto-ubiquitin transfer is unlikely to be the mechanism by which auto-ubiquitination enhances Mdm2's ability to stimulate p53 ubiquitination.

2.5. Mdm2 autoubiquitination with tagged-ubiquitin inhibits E3 activity

Initially, we had attempted to utilize differentially epitope-tagged ubiquitin for *in vitro* Mdm2 autoubiquitination versus p53 ubiquitination. The rationale was to use a tagged ubiquitin (HA, 6x-His or FLAG) for Mdm2 autoubiquitination and untagged ubiquitin for modifying p53, enabling the distinction between transfer and *de novo* synthesis of a ubiquitin chain. After the ubiquitination reactions, p53 would be immunoprecipitated and analyzed by western blot using an antibody against the

epitope tag on ubiquitin (ex: anti-HA). If p53 were being modified with tagged-ubiquitin, it would suggest a mechanism of auto-ubiquitin chain transfer onto the substrate. Unfortunately, the experiment could not be performed due to a major pitfall of using epitope-tagged ubiquitin. Intriguingly, the use of tagged-ubiquitin for autoubiquitination potently inhibited the E3 activity of Mdm2 (**Figure 2.9**). The greatest inhibitory effect stemmed from autoubiquitination with HA-tagged ubiquitin, but Flag or 6X-His-tagged ubiquitin also significantly inhibited Mdm2 activity toward p53. To determine whether this effect was due to different efficacies of Mdm2 autoubiquitination, Mdm2 was autoubiquitinated with HA-ubiquitin for various durations. HA-ubiquitinated Mdm2 was compared with untagged-ubiquitin Mdm2. When Mdm2 was modified with HA-ubiquitin even further, inhibition of p53 ubiquitination was even more dramatic (**Figure 2.10**). Many other studies in the ubiquitination field have utilized tagged-ubiquitin for their *in vitro* ubiquitination reactions and this observation may hint at a serious technical caveat when studying mechanisms of ubiquitination using tagged ubiquitin.

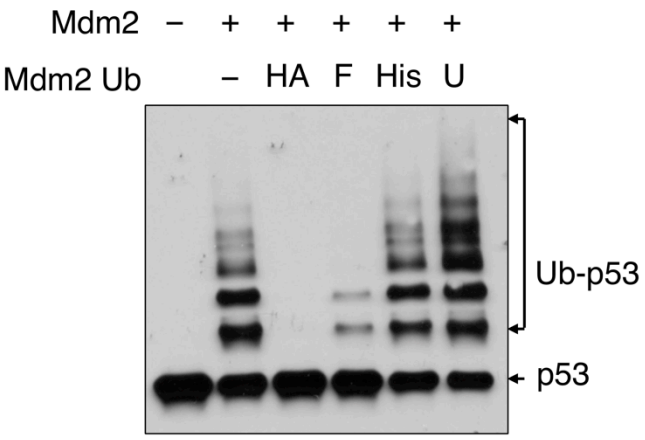

Figure 2.9. Epitope-tagged ubiquitin inhibits Mdm2 E3 activity.
Immobilized GST-Mdm2 was unmodified or autoubiquitinated with either untagged (U) or HA- (HA), Flag- (F), and 6x-His-tagged (His) ubiquitin. For p53 ubiquitination, p53 was incubated in the presence (+) or absence (-) of Mdm2 as indicated then analyzed by western blot with anti-p53 antibody.

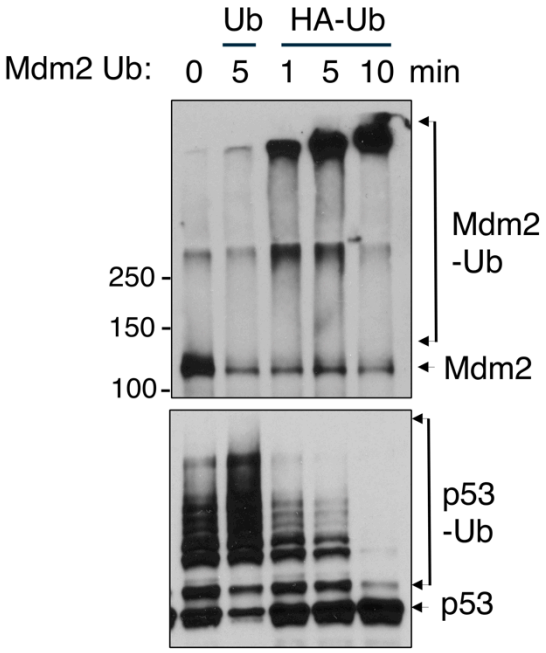

Figure 2.10. HA-tagged ubiquitin inhibits Mdm2 E3 activity.
Top: Immobilized GST-Mdm2 was unmodified (-) or autoubiquitinated with untagged ubiquitin (Ub) or HA-tagged ubiquitin (HA-Ub) for the indicated times and western blotted with anti-Mdm2. Bottom: p53 was ubiquitinated by unmodified or autoubiquitinated Mdm2 for 5 minutes and analyzed by western blot with anti-p53 antibody.

2.6. Auto-ubiquitination facilitates the interaction of Mdm2 with the UbcH5 E2 enzyme

Members of the UbcH5 family, which are the cognate E2s for an array of E3s including Mdm2, contain a ubiquitin-binding domain (UBD). The UBD is required for the processivity of UbcH5-mediated auto-ubiquitination of the RING domain ligase BRCA1. We reasoned that the poly-ubiquitin chains on Mdm2 could simultaneously recruit multiple E2∼Ubs through binding to their UBDs, thereby circumventing the requirement for multiple rounds of E2 recharging. To test this possibility, we compared the interactions of ubiquitinated and unmodified Mdm2 with recombinant UbcH5c (**Figure 2.11**, A) in an *in vitro* pull-down assay. Because of the transient nature of the E2-E3 interaction, we employed chemical cross-linking to stabilize the binding. The interaction of UbcH5c with ubiquitinated Mdm2 could be readily detected under these conditions, but the interaction between UbcH5c and unmodified Mdm2 could not (**Figure 2.11**, A lanes 1-3). In accordance with the lack of a stimulating effect of mono-ubiquitination on Mdm2's substrate E3 activity (**Figure 2.7**), the interaction between UbcH5c and mono-ubiquitinated Mdm2 could not be detected under these conditions (**Figure 2.11**, B). These results suggest that poly-ubiquitin chains on Mdm2 may enhance the recruitment of E2 enzymes.

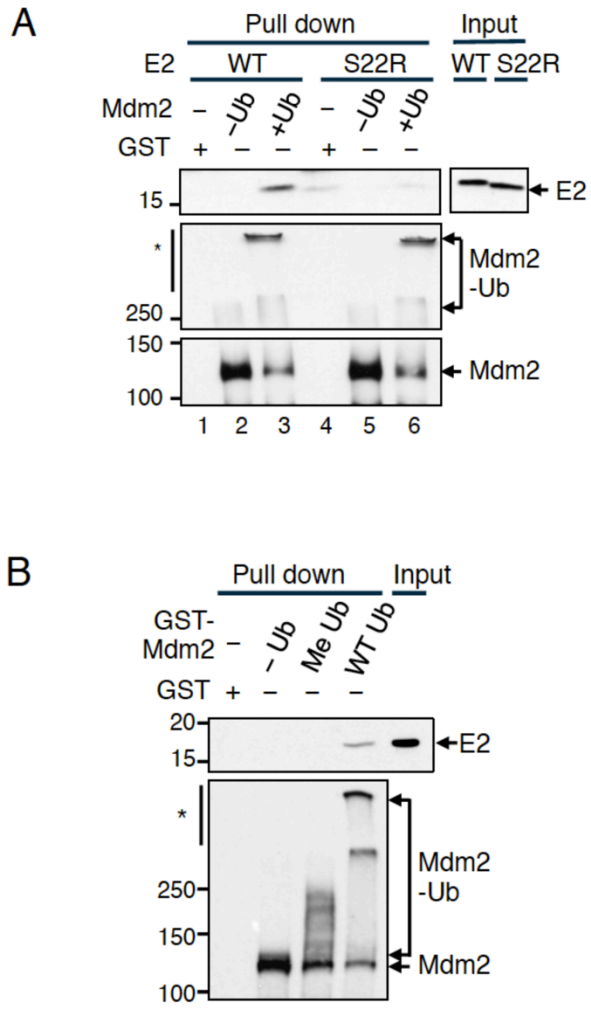

Figure 2.11. Mdm2 autoubiquitination enhances recruitment of UbcH5c. (A) In vitro binding of WT UbcH5c or S22R UbcH5c with GST, unmodified GST-Mdm2, or auto-ubiquitinated GST-Mdm2 with minimal reversible crosslinking. Input is 1% of total UbcH5c used for binding. Western blot analyzed with anti-UbcH5c (top) and anti-Mdm2 (middle and bottom). (B) In vitro binding of UbcH5c with GST (lane 1), unmodified GST-Mdm2 (lane 2), mono-ubiquitinated GST-Mdm2 (lane 3), or poly-ubiquitinated GST-Mdm2 (lane 4) with minimal crosslinking. Input is 0.5% of total UbcH5c used for binding. Immunoblotted with anti-UbcH5c (top) and anti-Mdm2 (bottom).

2.7. The non-covalent interaction between the UBD on E2s and ubiquitin is required for the enhancement of E2 recruitment and substrate E3 ligase activity of Mdm2.

To examine the functional importance of the non-covalent E2-ubiquitin interaction, we used E2 and ubiquitin mutations that impair the non-covalent interaction. Mutation of Ser22 within the UBD to Arg (S22R) impaired the interaction of UbcH5c with ubiquitin, while it did not affect the overall structure of UbcH5c or its thioesterification with ubiquitin (**Figure 2.12**, A). Unlike wild-type (WT) UbcH5c, the UbcH5c S22R mutant showed no enhanced binding to auto-modified Mdm2 (**Figure 2.11**, lanes 4-6). Moreover, in the presence of S22R, auto-modified Mdm2 became ineffective at conjugating p53 with WT ubiquitin (**Figure 2.12**, B) and even less effective at conjugating p53 with Lys48-only ubiquitin (**Figure 2.5**, lanes 8-13).

Most UBDs contact a hydrophobic surface on ubiquitin that is centered on Ile44 (Beal et al. 1998). Mdm2 conjugated with I44A ubiquitin showed no increase in binding to UbcH5c (**Figure 2.13**). We performed Mdm2 autoubiquitination using either I44A ubiquitin for different times (30 and 45 min), or WT ubiquitin for a shorter time (10 min) (**Figure 2.14**, A). Mdm2 conjugated with I44A ubiquitin showed noticeably reduced activity compared to Mdm2 conjugated with WT ubiquitin, especially at early time points (2 and 5 min) (**Figure 2.14**, B). Mdm2 with longer I44A ubiquitination (45 min) had even less activity compared to Mdm2 with

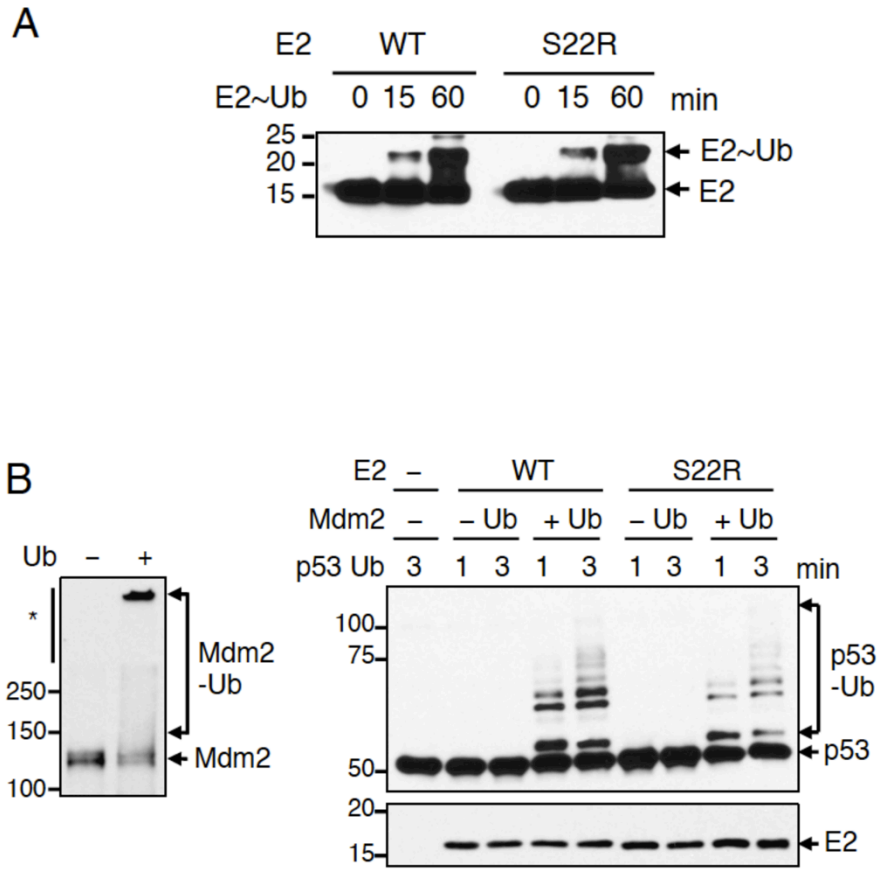

Figure 2.12. The UbcH5c S22R mutant renders auto-ubiquitination ineffective in stimulating Mdm2's substrate E3 activity.
(A) WT and S22R UbcH5c was thioesterified with ubiquitin for different durations. The reaction was analyzed by non-reducing SDS-PAGE and Western blot with anti-UbcH5c antibody. (B) GST-Mdm2 was unmodified (-) or auto-ubiquitinated (+) with WT UbcH5c and western blot was analyzed with anti-Mdm2 antibody (left). Ubiquitination of p53 by unmodified or auto-ubiquitinated Mdm2 in the presence or absence of WT or S22R UbcH5c analyzed with anti-p53 (top) and anti-UbcH5c (bottom).

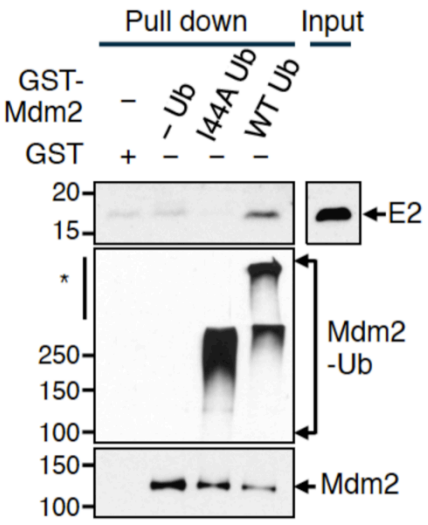

Figure 2.13. Ubiquitin mutant I44A impairs E2 recruitment.
In vitro binding of UbcH5c with GST, unconjugated Mdm2, or Mdm2 conjugated with I44A or WT ubiquitin. Input is 0.5% of total UbcH5c used for binding. Western blot was analyzed with anti-UbcH5c (top), anti-ubiquitin (middle), anti-Mdm2 (bottom).

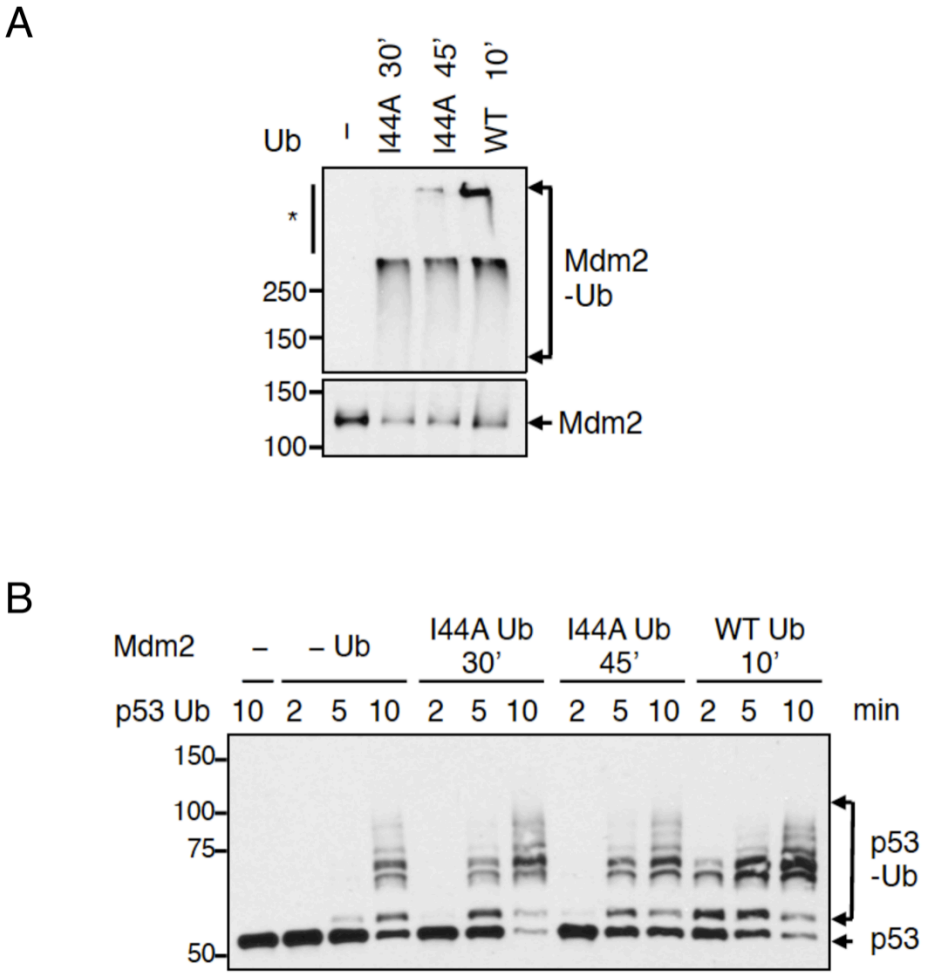

Figure 2.14. Ubiquitin mutant I44A impairs the substrate E3 activity of autoubiquitinated Mdm2.
Unconjugated Mdm2, or Mdm2 conjugated with I44A or WT ubiquitin for the indicated times (B) were used to ubiquitinate p53 for different durations (C). Mdm2 autoubiquitination was analyzed with anti-ubiquitin (top) and anti-Mdm2 (bottom). Ubiquitination of p53 was probed with anti-p53.

shorter I44A ubiquitination (30 min). Therefore, when the E2 and ubiquitin chains on Mdm2 cannot bind to each other, autoubiquitination becomes ineffective at stimulating Mdm2's substrate E3 activity.

2.8. Mdm2 affinity for thioesterified ubiquitin-conjugating enzyme

Our previous experiments showed that autoubiquitination increases Mdm2 affinity for the UbcH5c E2 enzyme. However, we utilized uncharged (non-thioesterified) E2 for all the previous experiments. To test whether autoubiquitination affects Mdm2 interaction with thioesterified E2, I performed E2 thioesterification and analyzed with non-reducing SDS-PAGE and western blot (**Figure 2.15**, A). Ubiquitin-charged E2 enzyme (E2~Ub) was then incubated with immobilized GST, unmodified GST-mdm2 or autoubiquitinated GST-mdm2 as described previously in Chapter 2.6. After minimal DSP crosslinking was performed, bound proteins treated with reducing agents to reverse crosslinks and analyzed by western blot for UbcH5c and Mdm2 (**Figure 2.15,** B). Intriguingly, the use of E2~Ub enabled the detection of E2 interaction with unmodified Mdm2 which could not be detected using uncharged E2 enzyme. There was however, no significant difference in E2~Ub binding between unmodified and autoubiquitinated Mdm2. It is possible that the difference is too minute to be detected under these assay conditions.

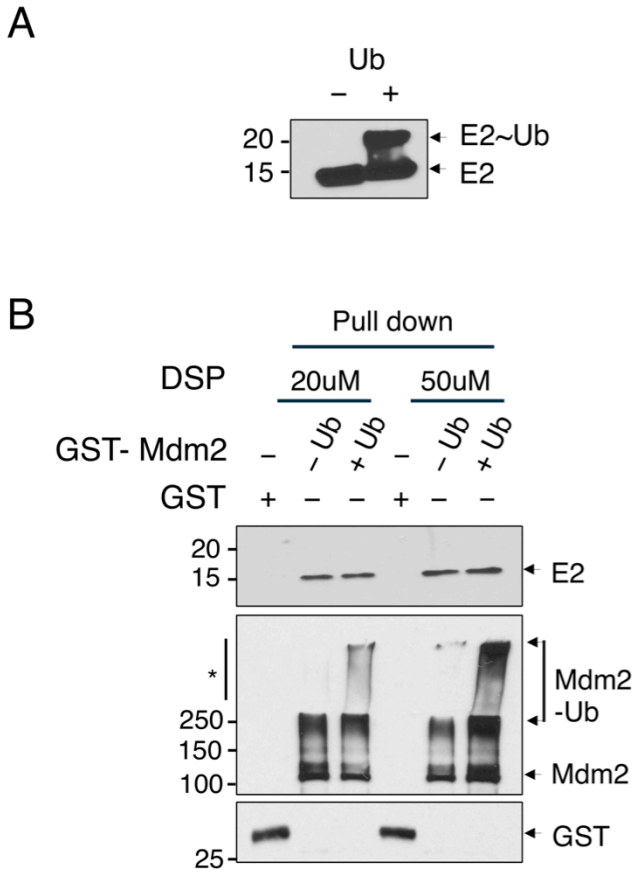

Figure 2.15. Mdm2 *in vitro* binding with thioesterified E2 enzyme.
(A) UbcH5c was thioesterified with ubiquitin. (B) Thioesterified ubiquitin was incubated with immobilized GST, unmodified GST-mdm2 (- Ub) or autoubiquitinated GST-mdm2 (+ Ub) followed by minimal DSP crosslinking at indicated concentrations. Bound proteins were analyzed by western blot with anti-UbcH5c and anti-GST antibodies.

CHAPTER 3 : BRG1 Regulates Mdm2 Stability as a Daxx-HAUSP Binding Partner

The tumor suppressor p53 plays a critical part in protecting against cellular transformation. It is the most commonly mutated gene in human cancer, with almost a 50% mutation rate. Acting as a central sensor of multiple cellular stresses, p53 can induce potent anti-proliferative outcomes through transcriptional control of hundreds of target genes. Activation of p53 can lead to reversible outcomes such as cell cycle arrest and DNA repair or the irreversible fates of senescence and apoptosis.

In an unstressed cell, intricate regulatory mechanisms are essential to allow cell survival and growth by restraining the potent activities of p53. The major negative regulator responsible for controlling p53 activity and stability is Mdm2, an E3 ubiquitin ligase. Mdm2 polyubiquitinates p53 and targets it for degradation by the proteasome. Mdm2 itself is also a short-lived protein due to rapid ubiquitination and degradation. Recent work from our laboratory suggests that Mdm2 is stabilized in a trimeric complex containing the adaptor protein Daxx and the deubiquitinating enzyme HAUSP (Herpes-associated ubiquitin-specific protease, also known as USP7). Daxx simultaneously interacts with Mdm2 and HAUSP, to bring them into close proximity. HAUSP then utilizes its deubiquitinase activity and removes ubiquitin molecules from Mdm2, to reduce Mdm2 ubiquitination and prevent its degradation. The Mdm2-Daxx-HAUSP complex is disrupted by phosphorylation events mediated via DNA damage signaling (Tang et al. 2013) and by the tumor suppressor RASSF1A (Ras-association domain

family 1 isoform A) (Song et al. 2008). The Mdm2-Daxx-HAUSP complex might represent a pivotal regulatory switch to govern the activation of p53. However, we still have a poor understanding of the assembly and composition of the Mdm2-Daxx-HAUSP complex.

Here we show that the SWI/SNF ATPase component, BRG1 is a novel binding partner of the Mdm2-Daxx-HAUSP complex. Dr. Yide Mei, a postdoctoral fellow in our laboratory and co-first author on the manuscript, initiated this study. I performed several key experiments, some in response to reviewers' comments. The study would not have been possible without reagents and cell lines provided by Dr. David N. Reisman, Associate Professor of Medicine at the University of Florida. We find that BRG1 interacts with Mdm2, Daxx and HAUSP and enhances assembly of the complex. I show that BRG1 demonstrates direct and specific interaction with Daxx and HAUSP *in vitro.* We show that BRG1 is essential for maintaining Mdm2 stability and I find this stabilization is independent of BRG1 ATPase activity. Morever, BRG1 controls cell proliferation, senescence and anchorage-independent growth via Mdm2 stabilization and p53 inhibition. These results uncover BRG1 as a crucial component of the Mdm2-Daxx-HAUSP complex and reveal a function for BRG1 that is required for cell survival, beyond its role as a chromatin remodeler.

3.1. BRG1 is a HAUSP- and Daxx-interacting protein

BRG1 was found to interact with HAUSP in an affinity purification experiment performed by Dr. Yide Mei. Flag-tagged HAUSP was expressed in an osteosarcoma cell line (U2OS) and cells were crosslinked with formaldehyde to stabilize protein-protein

interactions. Cell lysates were immunoprecipitated with anti-Flag beads and bound proteins were analyzed by mass spectrometry. BRG1 peptides were detected in Flag-HAUSP immunoprecipitates but not the control. In another independent interaction screen, BRG1 was also isolated as a Daxx-interacting protein. The interactions between overexpressed and endogenous BRG1 with Daxx and HAUSP were confirmed via co-immunoprecipitations in several cell lines. Representative immunoprecipitation results from overexpressed and endogenous proteins are shown (**Figure 3.1** and **3.2** respectively).

In order to test whether BRG1's interactions with each component of the Mdm2-Daxx-HAUSP complex was direct, I performed *in vitro* binding assays. I individually expressed HA-tagged HAUSP, GST-tagged Mdm2, or GST-tagged Daxx in HEK 293T cells. Each tagged protein was then purified and immobilized with anti-HA or Glutathione beads. As negative controls, each bead type was coated with lysate expressing the epitope tags alone. Flag-tagged BRG1 was also expressed and purified from HEK 293T cells using anti-Flag beads and eluted with 3x-Flag-peptide. Flag-BRG1 was incubated with each bead-immobilized protein and bound proteins were analyzed by coomassie staining, followed by Silver Plus staining (**Figure 3.3**). HAUSP bound a significant amount of the BRG1 input, suggesting that BRG1 can directly interact with HAUSP *in vitro* (**Figure 3.3**, A). Daxx also specifically bound a large portion of the BRG1 input but Mdm2 did not demonstrate a specific interaction, as its binding profile was comparable to that of the GST-only control (**Figure 3.3**, B). These results indicate that although Daxx and HAUSP can directly interact with BRG1, the interaction with Mdm2 maybe bridged by Daxx or HAUSP *in vivo.*

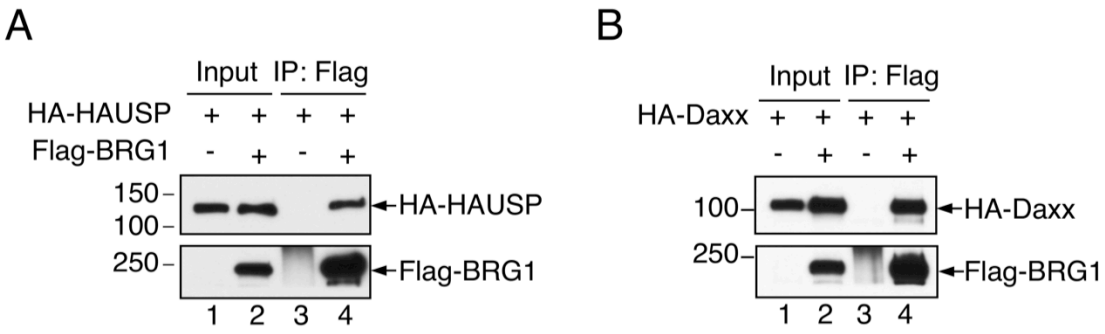

Figure 3.1. Overexpressed BRG1 co-immunoprecipitates with HAUSP and Daxx.
(A) HEK293T cells were transfected with HA-HAUSP alone or together with Flag-BRG1. Lysates were subjected to immunoprecipitation with anti-Flag antibody. The immunoprecipitates and input lysates were analyzed by western blot. Molecular weight standards (in kDa) are shown on the left. (B) Lysates from HEK293T cells expressing HA-Daxx alone or HA-Daxx plus Flag-BRG1 were subjected to immunoprecipitation with anti-Flag antibody. Figure courtesy of Dr. Yide Mei.

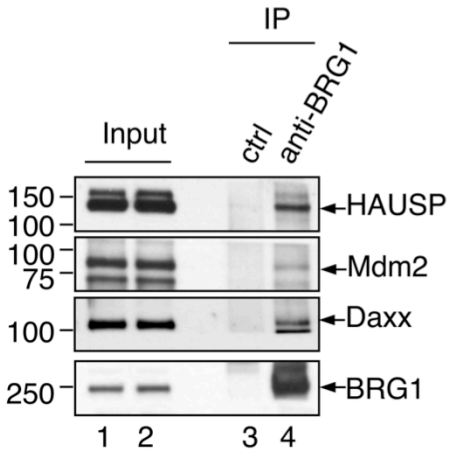

Figure 3.2. BRG1 co-immunoprecipitates endogenous Mdm2, Daxx and HAUSP. Lysates from U2OS cells were immunoprecipitated separately with anti-BRG1 antibody and a control rabbit antibody followed by western blot. Figure courtesy of Dr. Yide Mei.

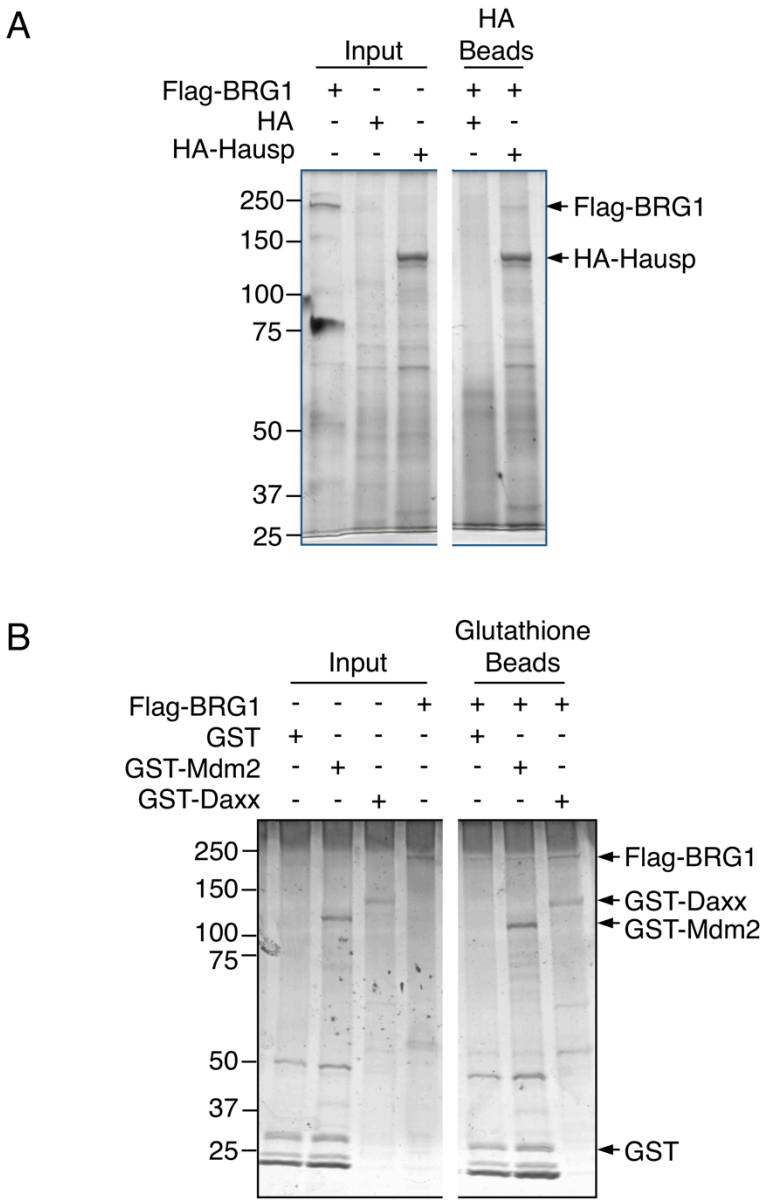

Figure 3.3. *In vitro* binding assay with BRG1, Daxx, HAUSP, and Mdm2. Purified Flag-BRG1 was incubated individually with Daxx, HAUSP or Mdm2 immobilized on beads. Bound complexes were resolved by SDS-PAGE and analyzed by silver stain. (A) BRG1 binding with HAUSP. (B) BRG1 binding with Daxx and Mdm2.

3.2. BRG1 enhances the assembly of the Mdm2-Daxx-HAUSP complex

To explore the role of BRG1 in the Mdm2-Daxx-HAUSP complex, Dr. Mei assessed its contribution to the binary interactions between Mdm2, Daxx and HAUSP. U20S cells were transduced with lentivirus expressing either control or BRG1-directed shRNA. Cells were transfected with HA-HAUSP and immunoprecipitated with anti-HA beads. Significantly less Mdm2 and Daxx co-immunoprecipitated with HAUSP in the BRG1 knockdown cells compared with control shRNA expressing cells (**Figure 3.4**). Thus, BRG1 depletion strongly reduced both HAUSP-Mdm2 and HAUSP-Daxx interactions. A sequential immunoprecipitation also confirmed that BRG1 is in a ternary complex with HAUSP and Mdm2 (**Figure 3.5**).

3.3. BRG1 plays a role in maintaining the stability of Mdm2

To determine whether BRG1 regulates Mdm2 protein stability, BRG1 expression was silenced using siRNA in *p53* wild-type U2OS cells and a pair of isogenic colon cancer HCT116 cells that are either *p53* wild-type or null (**Figure 3.6**, A). Knockdown of BRG1 expression reduced the steady state levels of Mdm2. Furthermore, half-life experiments under the protein translation inhibitor cycloheximide showed that BRG1 depletion reduced Mdm2 half-life while BRG1 overexpression lengthened the half-life of co-transfected Mdm2 (data not shown). When proteasomal degradation of Mdm2 was blocked by MG132 (proteasome inhibitor), Mdm2 levels were elevated. BRG1 no longer enhanced the levels of Mdm2 under MG132 treatment, suggesting that BRG1 prevents proteasomal degradation of Mdm2.

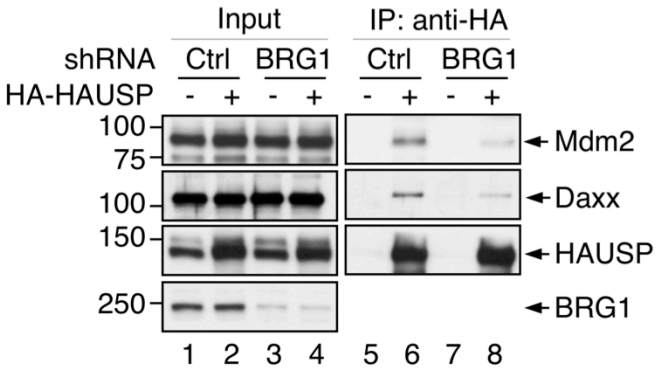

Figure 3.4. BRG1 knockdown affects Mdm2-Daxx-HAUSP complex formation. U2OS cells expressing BRG1 shRNA or control shRNA were transfected with or without HA-HAUSP. Cells were treated with MG132 for 6 h, and cell lysates were subjected to immunoprecipitation with anti-HA antibody conjugated on beads, followed by western blot. Figure courtesy of Dr. Yide Mei.

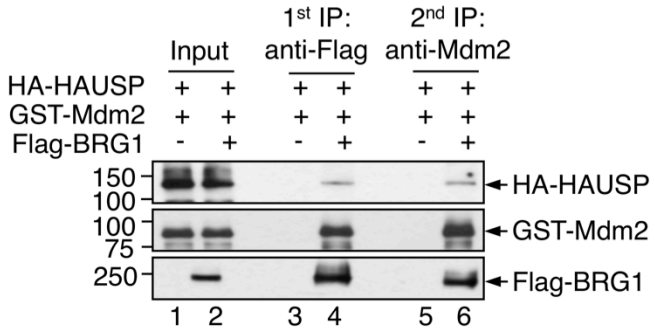

Figure 3.5. BRG1 is in a complex with Mdm2 and HAUSP.
HA-HAUSP and GST-Mdm2 were transfected into *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEF cells in the presence or absence of Flag-BRG1. Cells were treated with MG132 for 6 h. Lysates were first immunoprecipitated with anti-Flag antibody. Flag-BRG1 and the associated proteins were eluted with 3xFlag peptide. The eluents were subjected to another immunoprecipitation with anti-Mdm2 antibody. Figure courtesy of Dr. Yide Mei

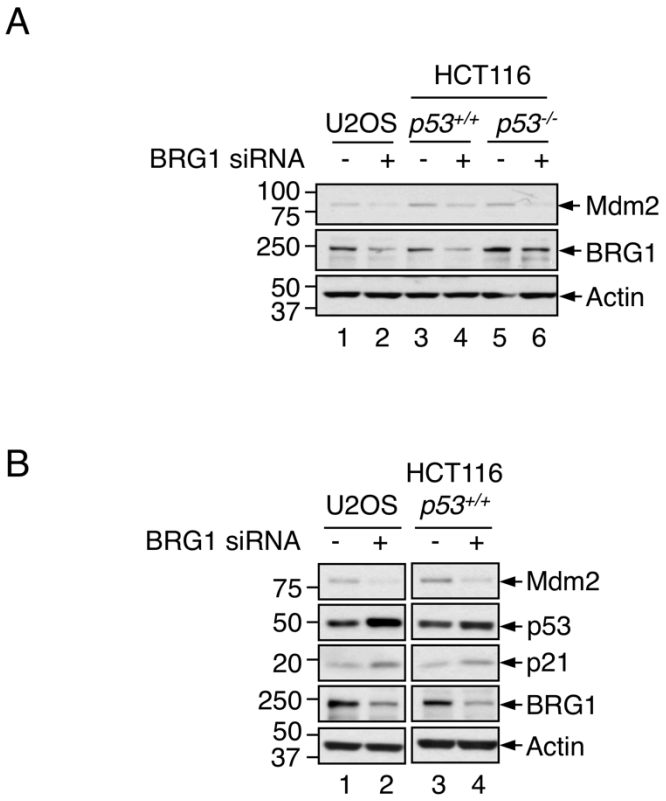

Figure 3.6. BRG1 regulates Mdm2 protein stability.
(A) U2OS, p53+/+ HCT116, and p53-/- HCT116 cells were transfected with BRG1 siRNA. 48 h after transfection, cells were harvested and cell lysates were subjected to western blot analysis with antibodies against indicated proteins. (B) U2OS and *p53*$^{+/+}$ HCT116 cells were transfected with BRG1 siRNA or a control siRNA. Cell lysates were analyzed by western blot with antibodies against indicated proteins. Actin served as a control sample loading. Figure courtesy of Dr. Yide Mei.

To examine whether BRG1 suppresses Mdm2 ubiquitination, Dr. Mei examined *in vivo* ubiquitination of Mdm2 in *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEF cells in the absence or presence of BRG1. Ubiquitination of Mdm2 was readily detected in the absence of BRG1, but BRG1 reduced Mdm2 ubiquitination strongly and in a dose-dependent manner (data not shown). Taken together, these results suggest that BRG1 stabilizes Mdm2 through blocking Mdm2 ubiquitination and degradation.

Upon siRNA-mediated BRG1 knockdown in U2OS and HCT116 cells, expression of p53 and one of its downstream targets, p21, was also increased (**Figure 3.6**, B). To confirm this result, I performed shRNA-mediated knockdown of BRG1 expression in U2OS cells and analyzed Mdm2 levels (**Figure 3.7**). As expected, BRG1 depletion with shRNA also led to a significant decrease in Mdm2. BRG1 is one of two mutually exclusive ATPase components in the SWI/SNF chromatin removeling complex. Knockdown of the alternative ATPase subunit, BRM, did not alter Mdm2 protein expression (**Figure 3.7**). Interestingly, BRG1 but not BRM knockdown, also led to an increase in p53 expression. Similar results were obtained when knockdown was performed in IMR90 primary human lung fibroblasts (data not shown).

These results suggest that BRG1 plays a crucial role in maintaining Mdm2 stability. To see whether BRG1 knockdown results in p53-mediated transcriptional activation of Mdm2, I analyzed the mRNA levels for *HDM2* in cells infected with control or BRG1 shRNA using quantitative real-time PCR (**Figure 3.8**). *HDM2* transcription is dramatically upregulated upon BRG1 knockdown. Remarkably, the western blot that I performed in parallel displays a reduction in Mdm2 protein expression confirming our previous observations on BRG1 knockdown compromising Mdm2 stability. This result

suggests that p53 is transcriptionally activated after BRG1 depletion due to the subsequent reduction in Mdm2. The increase in *HDM2* transcription is seemingly unable to compensate for the instability of Mdm2 protein in the absence of BRG1.

3.4. ATPase activity of BRG1 is not required for Mdm2 stabilization

BRG1 is a core component of the SWI/SNF remodeling complex and its ATPase activity is crucial for chromatin remodeling activity of the complex. We did not identify other complex components in either of our interaction screens for Daxx- or HAUSP-interacting proteins. We wanted to investigate whether BRG1 ATPase activity was important for Mdm2 stabilization. Mutation of a single conserved Lys residue to Arg (KR) within the C-terminal ATPase domain effectively renders human BRG1 ATPase defective (Khavari et al. 1993). Other groups have been referring to the mutant BRG1 as K798R, based on the amino acid position in the yeast homolog of BRG1. Previous studies have demonstrated that the KR mutant has a dominant negative effect when introduced with wild-type BRG1 because it may still retain the ability to interact with other SWI/SNF component (S. Bultman et al. 2000). I compared the abilities of wild-type and KR BRG1 to stabilize Mdm2, by expressing them in *BRG1*-null adrenal carcinoma cells (SW-13 cell line). Interestingly, expression of K798R stabilized Mdm2 in a dose dependent manner to an even greater extent than with wild-type BRG1 (**Figure 3.9**). This result suggests that BRG1 maintains Mdm2 stability independent of its chromatin remodeling activity. It is importance to note however that this cell line has mutant p53 status.

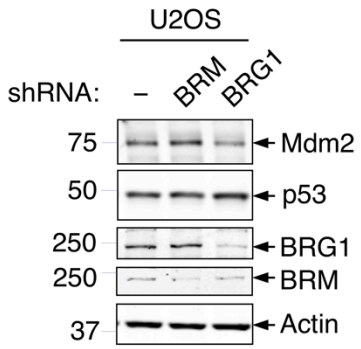

Figure 3.7. BRG1, not BRM, regulates Mdm2 stability.
U2OS cells were infected with lentivirus expressing a control shRNA, BRM shRNA, or BRG1 shRNA. Cells were harvested after 48 hours and cell lysates were analyzed by western blot.

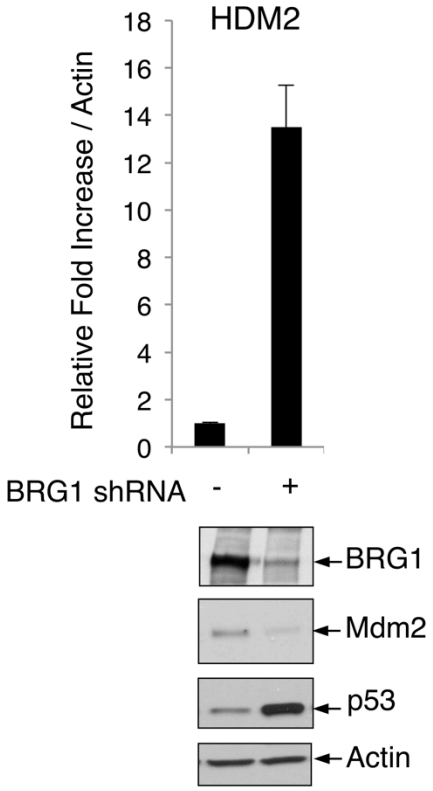

Figure 3.8. BRG1 silencing induces p53-mediated *HDM2* transcriptional activation.
U2OS cells were transduced with lentivirus expressing control shRNA or BRG1 shRNA. Cells were seeded in duplicate wells 24 hours after infection. 48 hours later cells were harvested for RNA extraction or lysate preparation. Results are shown for quantitative RT-PCR against human *Mdm2* (top) normalized against endogenous actin and standardized against the control shRNA group. Also shown is western blot analysis (bottom) for BRG1, Mdm2, and p53 with actin as a loading control.

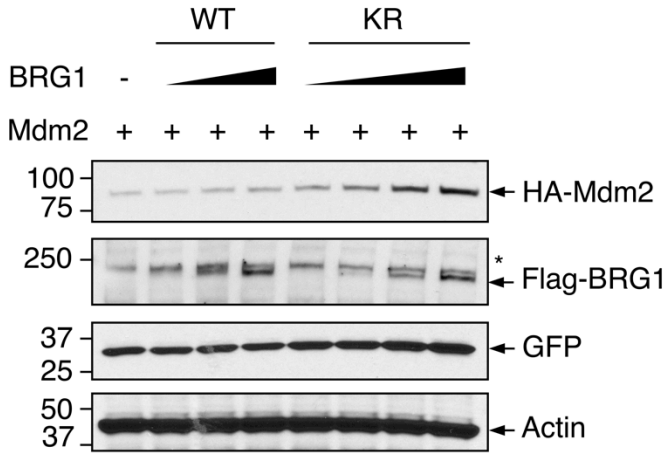

Figure 3.9. ATPase defective BRG1 can stabilize Mdm2.
BRG1-null SW13 cells were transfected with HA-mdm2, EGFP with or without BRG1-Flag (WT) or BRG1-K798R-Flag (KR). Cells lysates were analyzed with anti-HA^{HRP} antibody for Mdm2, anti-Flag antibody for BRG1. GFP is shown as a control for transfection efficiency while actin is used as a loading control.

3.5. BRG1 maybe a Mdm2 ubiquitination substrate

Mdm2 is a short-lived protein and its stability is inherently tied to the regulation of its own ubiquitination state. Mdm2 that is associated with MdmX, seems to preferentially ubiquitinate MdmX rather than subject itself to degradation. A prevailing thought in the field is that the increased stability of an E3 ligase in the presence of a potential substrate may be due to the diversion of its E3 activity away from the E3 towards the substrate. To test whether BRG1 contributes to Mdm2 stability by acting as an Mdm2 substrate, I first performed an *in vivo* ubiquitination experiment using N-terminal (1-650) BRG1 (NBRG1). Based on Dr. Mei's results, NBRG1 retains the ability to stabilize Mdm2. Due to the large molecular weight of BRG1, the use of this truncation of BRG1 simplifies the detection of ubiquitinated BRG1 product. Flag-NBRG1 and HA-tagged ubiquitin were expressed in *p53-/- mdm2-/-* MEFs either with or without GST-Mdm2 co-transfection. Cells were treated with proteasome inhibitor (MG132) to accumulate ubiquitinated protein, denatured and lysed before BRG1 was immunoprecipitated. Western blot analysis for ubiquitin conjugates demonstrates that there is a basal level of BRG1 ubiquitination even without Mdm2 (**Figure 3.10**). Interestingly, BRG1 ubiquitin conjugates are enriched in the Mdm2 co-expressing cells. This result suggests that N-terminal BRG1 may be ubiquitinated by Mdm2. However, it does not demonstrate BRG1 as a potential direct target of Mdm2. This observation could be due to an indirect effect of Mdm2 on another E3 ligase.

In order to test whether BRG1 could be directly ubiquitinated by Mdm2, I initially attempted to purify mammalian expressed BRG1 from HEK293T cells. However, due to possible contamination with a mammalian E3 ligase, I subsequently

purified recombinant NBRG1 from bacterial cells and performed *in vitro* ubiquitination with Mdm2. Incubation with GST-mdm2 produced ubiquitinated forms of BRG1 *in vitro* (**Figure 3.11**). This result confirmed that BRG1 could potentially act as a direct ubiquitination substrate of Mdm2.

3.6. BRG1 controls senescence, proliferation, and anchorage-independent growth in a p53-dependent manner

To determine the functional consequence of BRG1-mediated inhibition of p53, Dr. Mei evaluated the effect of BRG1 on cell proliferation, senescence, and anchorage-independent growth. BRG1 and p53 were knocked down individually or in combination in IMR90 human primary lung fibroblasts. Silencing of BRG1 alone led to a dramatic increase in the number of senescent cells (from ~2% to over 40%) (**Figure 3.12**, A). This effect was p53-dependent because simultaneous knockdown of p53 almost entirely abolished the increase in senescence (**Figure 3.12**, A). Knockdown of BRG1 also effectively inhibited IMR90 cell proliferation, which could be partially restored by the simultaneous silencing of p53 (**Figure 3.12**, B). To determine the effect of BRG1 on transformation, we performed a soft agar assay. This assay measures the ability of adherent cells to form colonies in an anchorage-independent manner, a hallmark of cancer cells. When BRG1 was silenced in U2OS cells, the number of colony-forming cells was reduced to less than half of those of the control cells (**Figure 3.13**). However, in p53 knockdown cells, which showed much enhanced ability to form foci in soft agar, knockdown of BRG1 no longer had an effect on the number of foci (**Figure 3.13**).

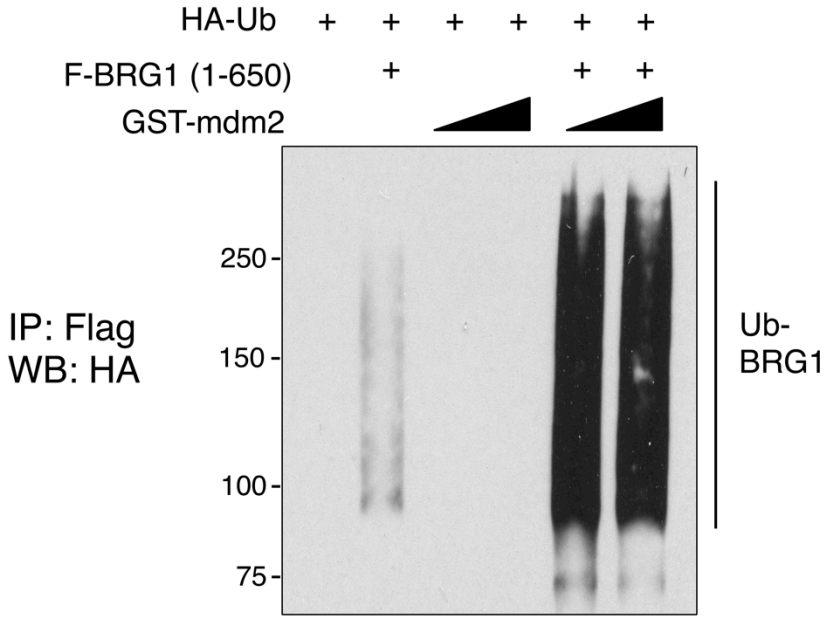

Figure 3.10. BRG1 *in vivo* ubiquitination.
DKO MEFs ($p53^{-/-}mdm2^{-/-}$) were transfected with Flag-BRG1 (1-650), GST-Mdm2 and HA-Ub where indicated and treated with MG132 for 4 hours. BRG1 was immunoprecipitated with anti-Flag beads and western blotted with anti-HA^{HRP} for ubiquitin-conjugates.

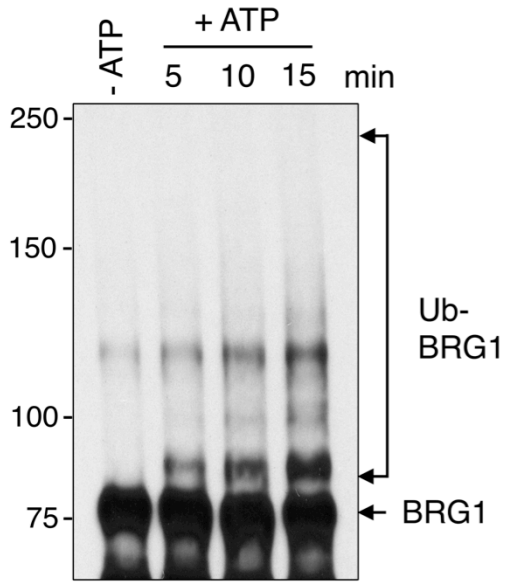

Figure 3.11. *In vitro* ubiquitination of N-terminal BRG1.
Flag-BRG1(1-650)-6xHis was expressed and dual-purified from bacterial cells. Recombinant BRG1 was ubiquitinated by incubating with immobilized GST-mdm2 at 37°C with or without ATP for the indicated times. Western blot was analyzed with anti-BRG1 antibody. *Non-specific band.

Taken together, these results suggest that BRG1 regulates cell senescence, proliferation, and anchorage-independent growth in a p53-dependent manner.

3.7. BRM can interact with Daxx and HAUSP

BRM is the alternative central ATPase subunit that is found in human SWI/SNF complexes. It is structurally similar to BRG1 and many studies indicate that BRG1 and BRM, although having distinct functional roles in a cell, can compensate for one another and have some overlapping functions. We did not identify BRM in our affinity purification screens for Daxx- or HAUSP-interacting proteins from U2OS cells. This does not preclude a role for BRM in regulating Mdm2 stability. The cell lines we utilized in our studies may have reduced BRM expression. I investigated whether BRM can associate with Daxx or HAUSP under overexpression in U2OS cells. Overexpressed BRM could co-immunoprecipitate Daxx and HAUSP respectively, indicating BRM could interact with either protein (**Figure 3.14**).

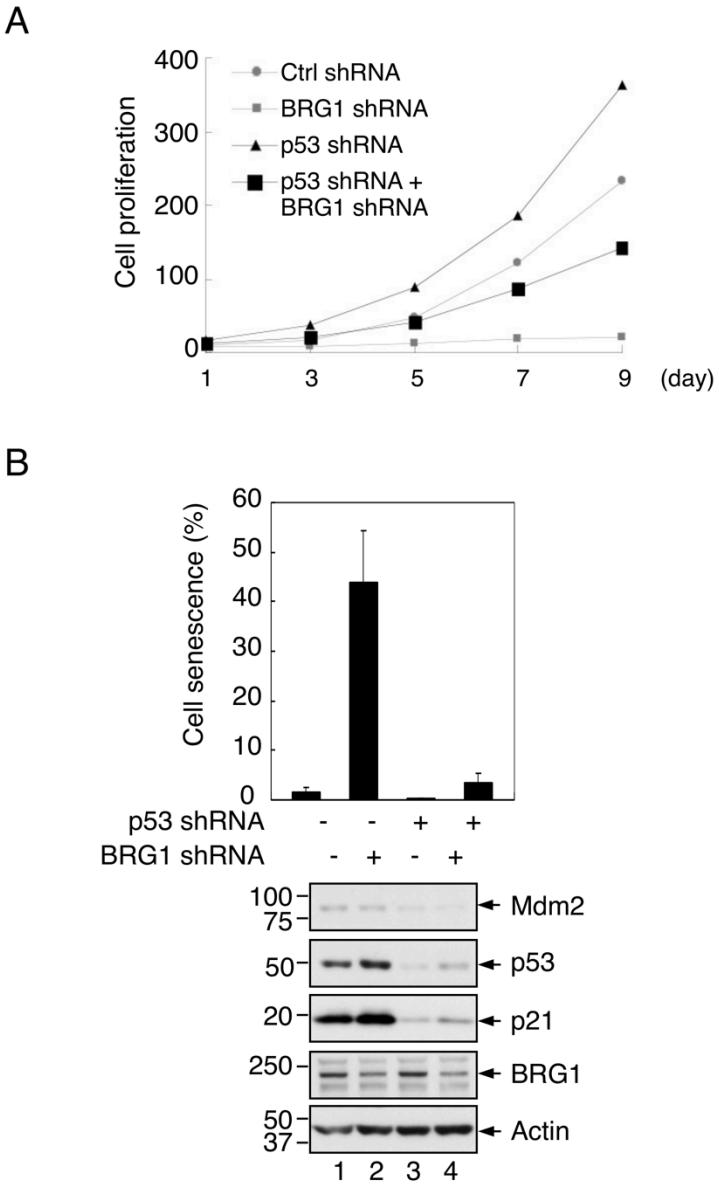

Figure 3.12. Cell proliferation and senescence assays.
(A) IMR90 cells were infected with lentiviruses expressing BRG1 shRNA, p53 shRNA, or both. 48 h after infection, cells were plated (day 0), and cell numbers were counted at the indicated time points. (B) IMR90 cells were infected with lentiviruses expressing the indicated shRNA(s). 96 h after infection, cells were stained for senescence-associated b-galactosidase activity. Cells were also harvested at 48 h after infection for analysis of protein expression. Figure courtesy of Dr. Yide Mei.

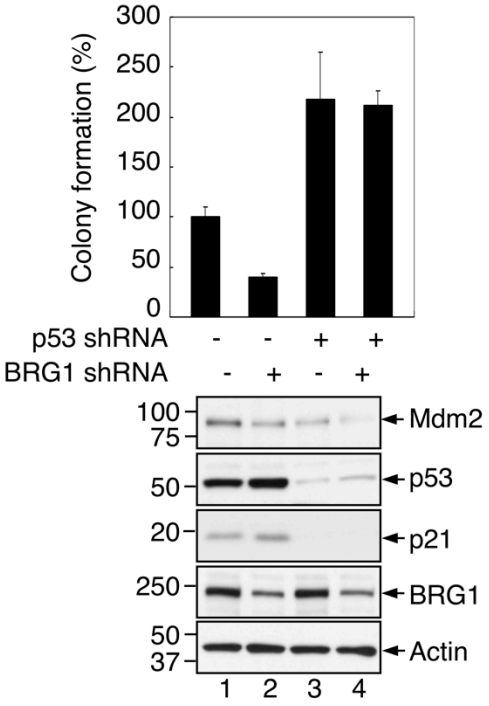

Figure 3.13. Anchorage-independent growth with BRG1 knockdown.
U2OS cells expressing control shRNA, BRG1 shRNA, p53 shRNA, or both BRG1 and p53 shRNAs were assayed for their ability to form the colonies in soft agar. For the colony formation assay, $1x10^4$ cells were used. The represented data are mean ± SD of three independent experiments. Figure courtesy of Dr. Yide Mei.

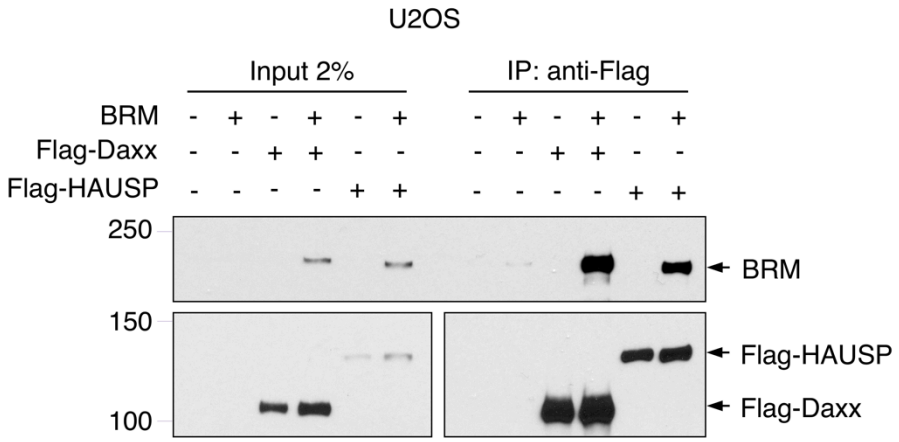

Figure 3.14. BRM can interact with Daxx and HAUSP.
U2OS cells were transfected with BRM, Flag-Daxx and Flag-HAUSP as indicated and cells were harvested 24 hours later. Daxx or HAUSP was immunoprecipitated with anti-Flag beads and analyzed by western blot with anti-BRM and anti-Flag antibodies.

CHAPTER 4 : Discussion and Future Directions

Mdm2 is the major negative regulator of the tumor suppressor p53. As a RING domain E3 ligase, Mdm2 targets p53 for ubiquitination and subsequent degradation by the proteasome. It is crucial to have multiple checks and balances to regulate the activity and stability of Mdm2. The mechanisms that enable processive ubiquitination of substrates by RING domain E3 ligases are also unclear. Autoubiquitination seems to be a general feature of RING E3s used to gauge the activity of the enzyme but its function remains overlooked. Data presented in Chapter 2 add to our understanding of an intricate autocatalytic mechanism to regulate the activation of the RING domain E3 ligase Mdm2.

Mdm2 has an extremely short half-life within a cell due to constant ubiquitination and degradation. A complex containing the adaptor protein Daxx and the deubiquitinase HAUSP mediate stabilization of Mdm2 and thus preventing p53 activation. In Chapter 3, evidence was presented for the role of the chromatin remodeling protein BRG1 in regulating Mdm2 stability and p53 activation by modulating the assembly of the Mdm2-Daxx-HAUSP complex.

In this chapter, how these findings impact our knowledge of how a RING E3 ligase functions and the regulation of Mdm2 are discussed. We propose mechanistic models based on our evidence to explain processive ubiquitination of p53 by Mdm2. We further discuss the role played by BRG1 in regulating the Mdm2-p53 axis.

4.1. Autoubiquitination of Mdm2 recruits multiple E2 enzymes to promote processive ubiquitination.

The data presented in Chapter 2 shows that autoubiquitination of Mdm2 results in an enhanced substrate ubiquitin ligase activity toward p53. Stimulation of E3 activity was dependent on the degree as well as the type of Mdm2 autoubiquitination. The extent of autoubiquitination affected the increase in E3 activity. Monoubiquitination of Mdm2 mediated by using methylated-ubiquitin was unable to stimulate E3 activity. Mechanistically, autoubiquitination of Mdm2 does not seem alter its affinity for p53. Enhancement of p53 ubiquitination does not seem to stem from direct ubiquitin "transfer" mechanism. Ultimately, examination of the UbcH5 and Mdm2 interactions uncovered a probable mechanism. Autoubiquitinated Mdm2 displayed higher affinity for UbcH5 enzymes with functional ubiquitin-binding domains (UBDs) but not mutant enzymes. Autoubiquitinating Mdm2 with I44A ubiquitin, unable to interact with E2 UBDs, compromised the stimulation of E3 activity as well as the UbcH5-Mdm2 interaction.

Altogether, this data suggests a model where the polyubiquitin chains on a RING domain E3 ligase act as "landing pads" for UbcH5~Ub recruitment through the non-covalent ubiquitin-UbcH5 interaction (**Figure 4.1**). The non-covalent ubiquitin-UbcH5 interaction has previously been shown to facilitate the self-assembly of UbcH5~Ub into multimeric complexes (Brzovic et al. 2006), which may further enrich UbcH5~Ub in the proximity of the E3-bound target protein. The increased local concentration of E2~Ub may overcome the rate-limiting step of E2 recruitment and permit processive ubiquitination of the substrate.

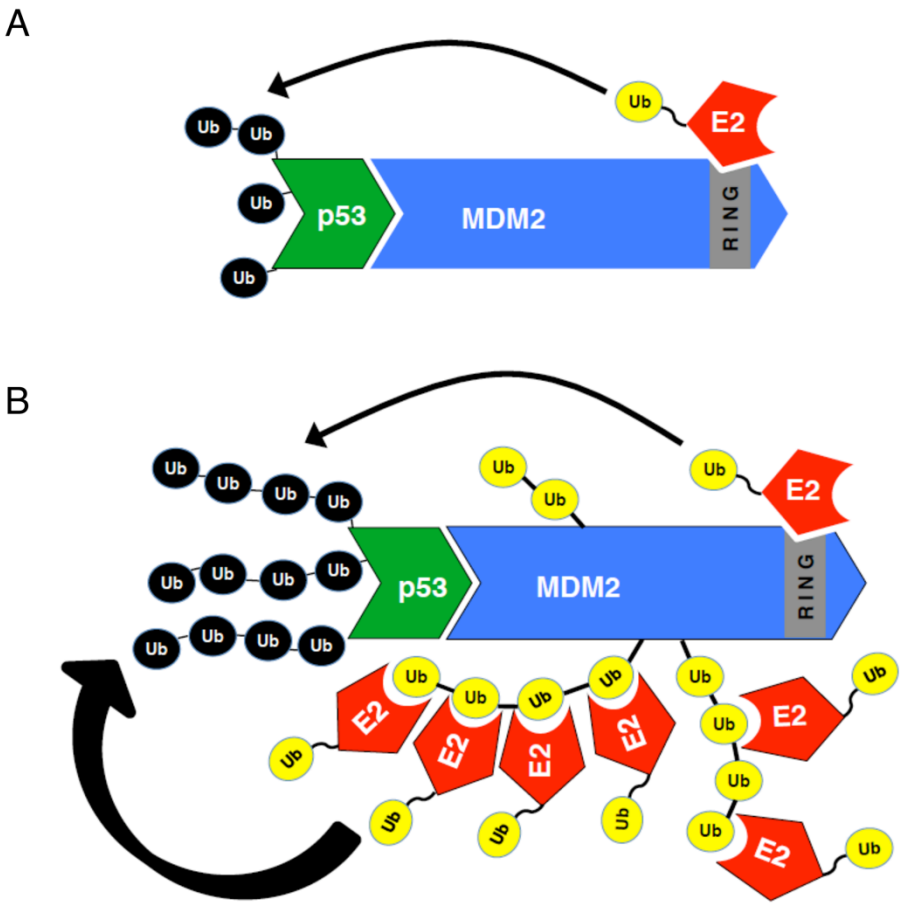

Figure 4.1. A model for enhancing Mdm2 substrate E3 activity.
(*A*) Unmodified Mdm2 recruits a single E2~Ub through the RING domain for each round of substrate ubiquitination. (*B*) The poly-ubiquitin chains on Mdm2 may act as "landing pads" to recruit multiple E2~Ub molecules via non-covalent interactions between ubiquitin and the UBD on E2s. The increased local concentration of E2~Ub molecules allows for processive ubiquitination of p53.

Structural biology studies demonstrate that an E3 ubiquitin ligase contains a shallow cleft on the surface of the RING domain that binds an E2 enzyme (Plechanovová et al. 2012). How the binding affinities of E2-E3 association contribute to ubiquitin ligase activity is unclear. The Brca1-Bard1 E3 can bind its partner E2 UbcH7 with tight affinity, but the pairing is inactive for ubiquitin transfer (Brzovic et al. 2003). In contrast, many other potent and highly active E2-E3 pairs do not display stable association, with dissociation constants in the low micromolar range (Deshaies and Joazeiro 2009). In terms of general enzyme function, this makes perfect sense. If an enzyme binds too tightly to a substrate, it would be difficult to carry out multiple rounds of catalytic activity.

This is consistent with our observations for the interaction between UbcH5 and Mdm2 (Chapter 2). We could only detect an association under the presence of chemical crosslinking, suggesting a low affinity transient interaction between the pair. However, it is important to realize that "naked" E2, not charged with ubiquitin, was used for many of the binding assays. When thioesterified UbcH5 (E2~Ub) was tested for interaction with Mdm2, there was no detectable difference between binding to unmodified and autoubiquitinated Mdm2 (**Figure 2.15**). It may well be that uncharged E2 enzymes have lower affinity for the RING domain of an E3 so they can discharge after the transferring their ubiquitin cargo. But maybe the discharged E2s continue to associate with the autoubiquitin chains on the E3 surface via non-covalent interactions. In contrast, the interaction of E2~Ub with Mdm2 was more readily detected in our assay, suggesting a much higher affinity for Mdm2's RING domain. It is possible that detectability of E2 recruitment via non-covalent interaction with ubiquitin was masked

by the strong interaction of E2~Ub with the RING domain. Recruiting an uncharged E2 enzyme to an autoubiquitinated E3 could be a solution to the problem of an E2's use of overlapping interfaces to interact with an E1 enzyme and the E3 RING domain. An E2~Ub bound to a RING domain, having completed ubiquitin transfer, must discharge in order to be recharged by the E1. The low affinity binding surfaces provided by autoubiquitin chains may enable on-site recharging of E2s by an E1, without complete disassociation from the E3. This model where an E2 enzyme has multidentate interactions with the E3 provides a solution to the rate-limiting step of E2 recruitment, enabling sequential assembly of a polyubiquitin chain on a given substrate.

Certain enzymes can be activated through autocatalytic action, as exemplified by the activation of receptor tyrosine kinases by autophosphorylation and of apoptotic proteases (caspases) by autoproteolytic cleavage. The results presented here further support the notion that autocatalytic action is a prevalent mechanism for switching on enzymatic activity. Like receptor tyrosine kinases and caspases, ubiquitin ligases catalyze a post-translational modification that has profound effects on various target proteins and that, if not controlled properly, can have deleterious consequences to the cell and the organism. Thus, it is vital to synthesize these enzymes with minimal or no activity, and to activate them in a controlled manner. Autocatalytic activation, as opposed to trans-activation by molecules of the same class or a different class of enzymes, would offer important advantages. It is highly efficient because of the reduced reliance on other enzymes. From an evolutionary point of view, autocatalytic activation might also be a necessity. When a new class of enzyme emerged, other regulatory proteins might not initially be able to perform the task, or might not even exist. Perhaps

more importantly, autocatalytic activation, as opposed to autocatalytic inhibition, engenders a built-in quality control mechanism: proteins that cannot fulfill the intended function would not become activated.

Outstanding Issues and Future Directions:

Autoubiquitination is a general feature of RING domain E3 ligases. At least two studies have previously uncovered the activating effect of RING E3 autoubiquitination. Polyubiquitination has been shown to enhance the E3 activity of the BRCA1/BARD1 ubiquitin ligase complex (Mallery, Vandenberg, and Hiom 2002). Modification of TRAF6 with Lys63-linked polyubiquitin is also an activating event (C. Wang et al. 2001). It is fair to speculate that autoubiquitination may also augment the activity of other multiple-domain RING ligases that use members of the UbcH5 family as their cognate E2s. In principle, autoubiquitination can accelerate other steps of ubiquitination and could be an activating event for multiple-domain RING ligases that employ E2s without an UBD. For Cullin-based RING ligases, the rapid E2-E3 association and dissociation, albeit facilitating substrate ubiquitination, cannot fully account for the high processivity of the reaction (Kleiger et al. 2009). It would be interesting to determine whether autoubiquitination also enhances the substrate E3 activity of enzymes belonging to the cullin-RING ligase family.

In optimizing this *in vitro* ubiquitination system, we have uncovered serious technical caveats that may have been previously overlooked. The use of epitope-tagged ubiquitin for ubiquitination assays is common in the literature. This may have produced erroneous results for other groups in the ubiquitination field. When either

HA, Flag, or 6x-His-tagged ubiquitin was used for Mdm2 autoubiquitination, the result was a dramatic inhibition of its substrate ligase activity (**Figure 2.9 and 2.10**). The inhibitory effect of tagged-ubiquitin was stronger with higher degrees of autoubiquitination. This inhibition may be due to the epitope tag disrupting the non-covalent interaction between the E2 UBD and ubiquitin. One can speculate that the presence of charged or hydrophobic residues in the tags may occlude the contact between Ile44 of ubiquitin and the E2 UBD. We may be able to use this observation as a guide to think of novel ways to target E3 activity using small molecules. Hypothetically, if tagged-ubiquitin conjugation inhibits E3 activity *in vitro,* targeting a small molecule *in vivo* could produce the same effect to inhibit aberrantly activated oncogenic E3 ligases. For example, it could prove to be an effective way to target tumors with Mdm2 overexpression.

Another point to consider in deciphering the dynamics of RING E3 activity is the ratio of enzyme to substrate. In our experiments, we maintained a 1:3 ratio of Mdm2 to p53 for ubiquitination reactions because the most distinctive difference in activity between unmodified and autoubiquitinated Mdm2 was seen under these conditions. The activity of an E3 ligase could potentially be regulated by the abundance of its specific substrate. In the case of Mdm2 and p53, their stoichiometric ratio in an unstressed cell is unknown. If p53 has some effect on Mdm2 autoubiquitination and its activity, degradation-resistant mutant p53 might be unable to do the same. It would helpful to have some idea of the stoichiometric ratios of p53 to Mdm2 under different cellular contexts.

Our results suggest a requirement of autoubiquitin chains, rather than monoubiquitin, on Mdm2 to enhance E3 activity. Further experiments should be performed to examine whether ubiquitin chains of different linkages can affect E3 activation. Moreover, whether specific sites of autoubiquitination can contribute differently to activity should be investigated. To that end, our laboratory has discovered three major ubiquitination sites on Mdm2 via mass spectrometric analysis (unpublished). We have mutated each lysine residue individually to arginine and plan to compare the activity of these mutants using assays described in Chapter 2. Interestingly, one of the sites lies within the RING domain while the others are outside the RING. In the three-dimensional structure of Mdm2, autoubiquitin chains situated close to or within the RING domain could be advantageous in recruiting E2 enzymes or altering RING domain conformation. With regard to sites of autoubiquitination, further studies should be performed to see whether HAUSP mediates site-specific or chain type-specific deubiquitination of Mdm2. So far, other E3 ligases including PCAF and Pirh2 have been implicated in targeting Mdm2 for ubiquitination. Studies should also focus on whether autoubiquitination sites on Mdm2 vary from those targeted by other E3 ligases.

As demonstrated for the receptor tyrosine kinases and for the precursors of caspase, autocatalytic activation can be induced by dimerization or oligomerization. Activation of Mdm2 is also likely induced by its homo-oligomerization or hetero-oligomerization with MdmX mediated by the RING domains on these proteins, especially the C-terminal amino acids of these domains. Mdm2 oligomers exhibit enhanced E3 activity compared to Mdm2 monomers, indicating an important role of

oligomerization in Mdm2 activation. Oligomerization also facilitates the autoubiquitination of Mdm2 or the Mdm2:MdmX complex. In a heterodimer formed by the RING domains of these proteins, self-ubiquitination occurs in *trans*, with Mdm2 ubiquitinating MdmX but not itself. The reasons for this selective ubiquitination is not completely clear, as the RING domains of Mdm2 and MdmX in this complex appear to adapt nearly identical structures. It is proposed that in an Mdm2 RING homodimer, one Mdm2 molecule might take on the role as a substrate, while the other one as the enzyme. Still, it is possible that in the complex formed by full-length Mdm2 or Mdm2 and MdmX proteins, autoubiquitination may occur in *cis*, as well as in *trans*. Also, the autoubiquitination may occur between different complexes instead of within the same complex. A precedent for the latter is shown for the activation of caspases, where the activating cleavage events occur between dimeric caspase precursors. This scenario would make autoubiquitination especially sensitive to the abundance of Mdm2.

Recent results from mouse models point to the importance of Mdm2:MdmX hetero-oligomerization for the ability of Mdm2 to restrain p53 in vivo. Mutation of the conserved cysteine residue in the MdmX RING domain, C462A, disrupts dimerization with Mdm2 and allows for p53 activation, leading to embryonic lethality by day 9.5. Notably, in the $MdmX^{C462A}$ mouse model, disruption of hetero-dimerization results in less Mdm2 autoubiquitination and higher levels of p53 and Mdm2. This result hints at a mechanism in which heterodimerization is crucial to activate Mdm2 through autoubiquitination. We envision a scenario where under physiological settings Mdm2 alone is unable to function as a potent E3 ligase probably due to its low abundance and the relatively weak self-association. In comparison, the Mdm2:MdmX association may

occur more readily, which triggers the formation of autoubiquitin chains that recruit multiple E2s to processively poly-ubiquitinate p53.

Regardless of the precise mechanism, the activation of RING domain ubiquitin ligases such as Mdm2 likely follows a similar mode to the oligomerization-induced activation of receptor tyrosine kinases and caspases. In this case, autoubiquitination likely rids the cell of excessive E3s when the concentration of an E3 reaches a threshold while no substrates are around, thereby allowing a homeostatic control of the levels of these ligases.

4.2. BRG1 is a scaffold maintaining Mdm2 stability and inhibiting p53 activation

Regulation of Mdm2 stability is particularly important for p53 regulation. The work presented in Chapter 3 reveals that BRG1, a core ATPase of the SWI/SNF chromatin-remodeling complex, plays a critical role in regulating Mdm2. It has been previously shown that HAUSP-mediated deubiquitination by the Daxx-HAUSP complex helps prevent Mdm2 degradation. Here, we find that BRG1 is a Daxx and HAUSP interacting partner and a crucial new component of the Mdm2-Daxx-HAUSP complex, functioning as a protein scaffold to bring the subunits together. Silencing BRG1 expression in cancer cell lines and primary cells led to a marked decrease in Mdm2 at the protein level as well as increase in p53 expression. The observed decrease in Mdm2 protein could be rescued by inhibiting the proteasome, suggesting that BRG1 prevents proteasomal degradation of Mdm2. Mdm2 half-life experiments in addition to *in vivo* ubiquitination assays confirmed that BRG1 is crucial for regulating ubiquitination-

mediated degradation of Mdm2. Intriguingly, the effects of BRG1 on Mdm2 stability are independent of its chromatin remodeling activity suggesting a function separate from the SWI/SNF complex. We also discover that BRG1 has the potential to be a substrate for Mdm2 ubiquitination. Through Mdm2 stabilization, BRG1 promotes p53 degradation and enhances cell growth and transformation. Based on our findings, a new model for the control of Mdm2 stability is proposed (**Figure 4.2**). BRG1 functions as a scaffold to house Daxx and HAUSP with Mdm2, enabling HAUSP-mediated deubiquitination of Mdm2. BRG1 itself may be subject to Mdm2-mediated ubiquitination, helping fine-tune the level of Mdm2 ubiquitination. Stabilized Mdm2 can efficiently ubiquitinate and target p53 for degradation. The functional relevance of BRG1-mediated p53 regulation is apparent in the BRG1 knockdown experiments demonstrating p53-dependent effects on proliferation, senescence and anchorage-independent growth.

BRM is the paralogous ATPase subunit found in human SWI/SNF complexes. Studies indicate that BRM is structurally similar to BRG1. Functionally, it is difficult to entirely separate BRM and BRG1. While BRG1 is preferentially expressed in proliferating cells, BRM is more often highly expressed in differentiated cells (Bourachot, Yaniv, and Muchardt 2003). Mouse models phenotypes of either protein show dramatic differences as well. BRM null mice develop normally whereas BRG1 deletion results in early embryonic lethality (S. Bultman et al. 2000; Reyes et al. 1998). There have been several studies that demonstrate the capability of BRG1 and BRM to compensate for the lack of the other, if expressed higher than normal. We found that Mdm2 levels were not affected with BRM knockdown compared with BRG1 depletion.

However, this was only tested in U2OS cells (osteosarcoma). Although it is possible that BRM does not contribute to Mdm2 stabilization, it is likely that this cell line has a much higher abundance of BRG1 than BRM. Moreover, BRM was capable of interacting with Daxx and HAUSP at least under overexpression in the same cell line.

Our experiments indicated that an ATPase defective BRG1 mutant could stabilize Mdm2. This finding suggests that the stabilizing effect of BRG1 on Mdm2 is independent of BRG1's chromatin remodeling function. However, this KR mutant has previously been characterized as a dominant negative in the presence of wild type of BRG1. It is unclear why this mutant form has a stronger effect on Mdm2 stabilization than the wild type.

BRG1 appears to regulate p53 at multiple levels. A recent study suggested that BRG1 also enhances CBP-mediated polyubiquitination of p53 (Naidu et al. 2009). The same study also confirmed our observation that BRG1 knockdown, but not BRM, could activate p53. Because BRG1 has a minimal effect on p53 in the absence of Mdm2, the effect of BRG1 on CBP may also be dependent on Mdm2. Consistent with this notion, previous studies have shown that CBP and Mdm2 cooperate in the ubiquitination of p53 (Ferreon et al. 2009). BRG1 likely provides an interaction surface that brings together multiple components of the Mdm2 complex, including CBP.

The identification of BRG1 as a crucial component of the Mdm2-Daxx-Hausp complex suggests a previously unappreciated complexity in Mdm2 regulation. The intricacy of the Mdm2 complex may enable fine-tuned regulation of p53 in response to various stresses. Because Daxx, HAUSP, and BRG1 are all involved in multiple cellular

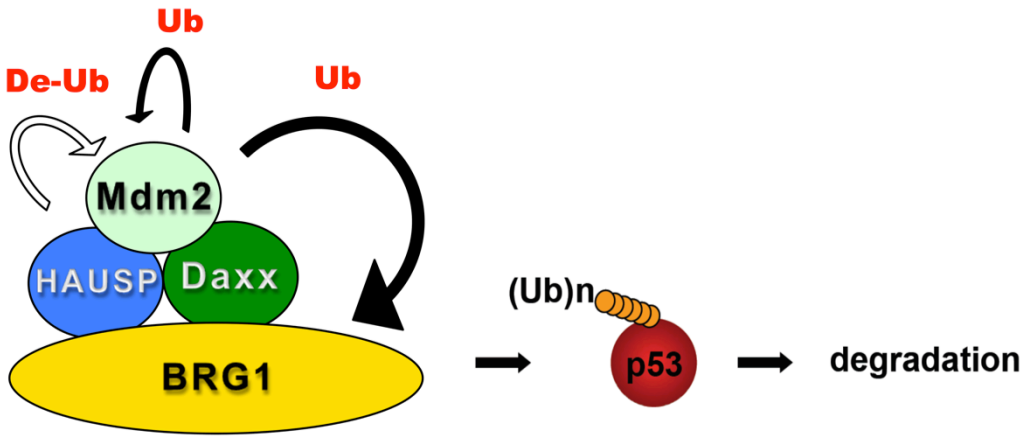

Figure 4.2. A model for the regulation of Mdm2 stability.
In an unstressed cell, BRG1 may function as a scaffold for the Mdm2-Daxx-HAUSP complex. Mdm2 autoubiquitination is controlled through deubiquitination by HAUSP. BRG1 may also be a substrate for Mdm2 ubiquitination. Mdm2 stability is maintained, enabling it to polyubiquitinate p53, targeting it for degradation by the proteasome.

processes including altering chromatin structures and proliferative signaling, they may link the perturbation of these processes to the activation of p53 via Mdm2.

Additionally, the predominant form of Mdm2 *in vivo* is as a heterodimer with MdmX. The heterodimeric Mdm2-MdmX complex is a more potent E3 ubiquitin ligase for p53. Dimerization with MdmX is also deemed important to maintain Mdm2 stability. Apart from facilitating HAUSP-mediated deubiquitination of Mdm2, BRG1 could provide an interaction surface to accommodate MdmX association with the multimeric complex involving Daxx, HAUSP and Mdm2. We may still be scratching at the surface in terms of the size and composition of this Mdm2-p53 regulatory complex.

Outstanding Issues and Future Directions

Our findings have uncovered the chromatin remodeler BRG1 as a novel partner of the Daxx-Mdm2-HAUSP complex. However, there are still many unanswered questions about how this complex regulates the Mdm2 and p53 axis.

The protein subunits of this tetrameric complex have been attributed many other functions related to gene regulation. Daxx has been characterized as a novel chaperone for the histone variant H3.3 (Drané et al. 2010) and as a regulator of multiple transcription factors (Salomoni and Khelifi 2006). Mdm2 has the ability to monoubiquitinate histone H2A and H2B (Minsky and Oren 2004) as well as recruit histone modifying proteins (Chen et al. 2010). Several studies have also found Mdm2 is bound to p53 on chromatin (Arva et al. 2005). It would be interesting to see whether BRG1, Daxx, Mdm2, and HAUSP regulate p53 as a chromatin-bound complex. Lastly, BRG1 as a chromatin remodeler and regulator of p53 through CBP (Naidu et al. 2009), is

bound to p53 target gene promoters. It is likely that the entire complex mediates both stability and transactivation of p53. Altogether, the Mdm2-Daxx-HAUSP-BRG1 complex contains a combination of enzyme activities and protein-interaction surfaces, analogous to a swiss-army knife. Mdm2 and HAUSP contribute ubiquitin ligase and deubiquitinase activities while Daxx and BRG1 contribute the ability to recognize acetylated histones (Shen et al. 2007) as well as protein-protein interaction domains capable of recruiting a multitude of transcription regulatory proteins. It would be exciting to determine whether these protein components work together to keep p53 in a transcriptionally inactive state either by mediating other post-translational modifications of p53 or by affecting histone modifications at the target promoters.

Given that the ATPase activity of BRG1 is not involved in Mdm2 stabilization, BRG1 likely provides a platform on which the other components of the Mdm2 complexes assemble. Because the absence of Daxx also diminishes the association between Mdm2 and HAUSP, BRG1 and Daxx may act together to cement interaction of Mdm2 with HAUSP. The ability of ATPase mutant BRG1 to stabilize Mdm2 does not preclude a role for SWI/SNF components in affecting Mdm2 stability. Further studies involving immunoprecipitations for the possible presence of SWI/SNF components in the Mdm2 complex are necessary. Structurally, BRG1 has multiple domains for mediating protein-protein interactions. Designing a BRG1 mutant that is unable to interact with SWI/SNF but maintains binding to Daxx and HAUSP could decipher whether regulation of Mdm2 is SWI/SNF independent. In addition to the SWI/SNF chromatin remodelers, BRG1 is present in heterogeneous complexes (Trotter and

Archer 2008). It would be of interest to determine whether BRG1 has a scaffolding role in some or all of these complexes.

Previous studies have established that the Mdm2-Daxx-HAUSP complex is disrupted by phosphorylation events due to DNA damage signaling (Tang et al. 2006, 2013). Additional experiments should be performed to assess whether BRG1 remains bound to Daxx and HAUSP upon DNA damage. Several recent discoveries indicate that BRG1 is recruited to multiple protein complexes during DNA damage signaling. At least one study demonstrates BRG1 recruitment during nucleotide excision repair (Zhao et al. 2009). Another makes a case for BRG1 requirement to promote phosphorylation events at DNA damage sites in order to recruit BRCA1 protein and mediate DNA repair (Zhang et al. 2013). Moreover, the interaction of BRG1 with CBP and with p53 target gene promoter was diminished upon treatment with doxorubicin, a DNA damaging agent (Naidu et al. 2009).

The critical role of BRG1 in the suppression of p53 via Mdm2 provides an explanation for its indispensible role in the survival of embryonic and adult tissues. It may also account for its high expression in proliferating cells (Glaros et al. 2008), where the suppression of p53 would be vital for growth. The requirement of BRG1 to restrain p53 may explain the abundance of *BRG1*-null cancer cell lines with mutant p53 (Naidu et al. 2009). BRG1-null cell lines retaining wild-type p53 are extremely rare. Despite this growth-promoting property, the enhanced tumor formation in BRG1 heterozygous mice indicates a tumor suppression function of BRG1 (S. J. Bultman et al. 2008). This is likely related to a compromised Rb pathway as BRG1 is required for the Rb-induced cell cycle arrest (Bartlett et al. 2011). BRG1 haplo-insufficiency may also result in genomic

instability due to defects in BRCA1-mediated DNA repair (Bochar et al. 2000; Zhang et al. 2013), further promoting tumor formation. Taken together, all of the studies on BRG1 highlight the complex role it plays cancer developed by regulating the activities of p53 and various other tumor suppressors. Future studies on the molecular mechanisms of BRG1 function in various cellular contexts will hopefully uncover a vulnerability that we can use to target specific cancer types.

CHAPTER 5 : Materials and Experimental Methods

5.1. Autoubiquitination of Mdm2 enhances its substrate ligase activity

Plasmids and reagents

Plasmids for expressing p53 and Mdm2 in mammalian cells are in pRK5 vector with N-terminal Flag, HA, or GST tags as described previously (Tang et al. 2006)T. UbcH5c WT pET28a (Plasmid 12643) and UbcH5c S22R pET28a (Plasmid 12644) (Brzovic et al. 2006)were obtained from Addgene (www.addgene.org).

The following reagents were purchased from Boston Biochem: ubiquitin E1 (E-305), UbcH5a (E2-616), Mg^{2+}-ATP (B-20), ubiquitin (U-100H), methylated ubiquitin (U-501), Lys48-only ubiquitin (UM-K480), and I44A ubiquitin (UM-I44A).

The antibodies for the following proteins were purchased from the indicated sources: p53 (DO-1, Santa Cruz Biotech.); Mdm2 (Ab-1, Calbiochem); ubiquitin (P4D1, Santa Cruz); poly-ubiquitinated conjugates (FK1 clone, Enzo Life Sciences); UbcH5 (A-615, Boston Biochem); UbcH5c (ab58251, Abcam); and MdmX (A300-287A, Bethyl Scientific).

Protein expression and purification

<u>Mdm2 and p53</u>

The corresponding expression plasmids were transfected into HEK293T cells. Cells expressing Mdm2 were further treated with proteasome inhibitor MG132 for 4 h. Cells were rinsed with ice-cold 1x PBS and lysed in Lysis Buffer (20 mM Tris-HCl pH7.4, 150

mM NaCl, 10% Glycerol, 1 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 1 mM DTT, 1 mM PMSF, 100 mM NaF, and 1x complete protease cocktail). GST-Mdm2 was precipitated with Glutathione Sepharose 4B beads (GE Healthcare, Cat. # 17-0756-01), and HA-Mdm2 with anti-HA affinity beads (Roche). Bead-bound Mdm2 was sequentially washed 2 x with Lysis Buffer, 1 x with Lysis Buffer plus 0.5 M KCl, 1 x with Lysis Buffer plus 1 M KCl, and 1x with ubiquitination reaction buffer. Bead-bound Mdm2 was re-suspended in ubiquitination reaction buffer and used for subsequent *in vitro* reactions. Flag-tagged p53 was purified with M2 beads (Sigma) as previously described (13) and eluted from the beads with Elution Buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM DTT, 10% Glycerol) plus 3xFLAG peptide (Sigma, Cat. # F4799).

Mdm2:MdmX complex

GST-Mdm2 and Flag-MdmX were co-expressed in HEK293T cells. Cells were treated with proteasome inhibitor MG132 for 4 h. Lysates were incubated with M2 beads for 3 h at 4 °C. Beads were washed 4 x with Lysis Buffer and 2 x with Elution Buffer. Bound MdmX was eluted with 3x-FLAG peptide for 1.5 h at 4 °C. Eluate was incubated with glutathione beads in Lysis Buffer overnight. Bead bound Mdm2:MdmX complexes were washed as described for the purification of Mdm2 proteins.

Recombinant WT and S22R UbcH5c

BL21 DE3 cells containing either WT UbcH5c pET28a or S22R UbcH5c pET28a were induced with 0.2 mM IPTG for 4 h at 30 °C. Cells were re-suspended in Sonication Buffer (20 mM HEPES, pH 6.0, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT) and lysed by

sonication. Lysates were centrifuged at 13,000 rpm for 15 minutes. Supernatant was fractionated by gel filtration using a Superdex 200 10/300 GL Column driven by an AKTA FPLC system (GE Healthcare). Fractions of 0.5 ml each were collected. Purified proteins were resolved by SDS-PAGE, stained by Coomassie, and quantified by densitometry against a BSA standard curve or by Western blot against known protein standards. Fractions containing only UbcH5c were pooled and used for ubiquitination/binding reaction.

Western blot

Proteins in sample buffer containing 5% 2-mercaptoethanol were boiled at 95 °C for 5 min and resolved by 8% SDS-PAGE for Mdm2 and p53, 15% SDS-PAGE for E2, and 8-15% gradient for simultaneously detecting GST and GST-Mdm2. Stacking gels were retained for all ubiquitination reactions. Proteins were transferred onto nitrocellulose membrane. For ubiquitin blotting, membranes were boiled in water for 2 min using a microwave prior to blocking. Membranes were blocked with 5% Non-fat Dry Milk in PBS-T and probed with indicated antibodies.

In vitro ubiquitination

Auto-ubiquitination reactions consisted of 3-5 ng bead-bound Mdm2, 100 nM E1, 500 nM UbcH5a, 2 mM Mg^{2+}-ATP, 2 mM DTT, and 2-5 µg wild-type or mutant ubiquitin in final volume of 20 µl Ubiquitination Reaction Buffer (40 mM Tris-HCl, pH 7.6). In control reactions, either ubiquitin (in Fig. 2*B*) or Mg^{2+}-ATP (in the rest of figures) was

omitted. Reaction mixtures were incubated at 37 °C on a microtube orbital shaker (Labnet, Shaker20) at 1,400 rpm and were either stopped by addition of sample buffer, or washed 3 x with Ubiquitination Reaction Buffer and aliquotted in separate tubes for p53 ubiquitination. p53 ubiquitination was performed at 22 °C with 10 ng Flag-p53 for 5 min or the indicated times. Mdm2 and p53 ubiquitination was detected by Western blot using anti-Mdm2 and anti-p53 antibody, respectively. To detect p53 poly-ubiquitination, Flag-p53 (30 ng) was ubiquitinated by Mdm2 as described above. Reaction mixtures were denatured by adding SDS to 1% final concentration and boiling for 5 min, and diluted to reduce the SDS concentration to 0.1%. Flag-p53 was pulled down with anti-flag M2 beads (Sigma) and analyzed by Western blot with anti-poly-ubiquitin or anti-p53 antibodies.

E2 Thioesterification

Thioesterification of E2 was performed using 150 nM E1, 600 ng WT or S22R E2, 100 mM NaCl, 5 mM Mg^{2+}-ATP, and 2 μg ubiquitin in a final volume of 20 μl Ubiquitination Reaction Buffer. Reactions were incubated at 22°C for indicated times and analyzed by non-reducing SDS-PAGE and Western blot.

In vitro binding assays

For p53 and Mdm2 binding, GST-Mdm2 (unmodified or auto-ubiquitinated) immobilized on glutathione beads was first blocked with 3% BSA for 1 h at 4 °C. Beads were incubated with 30 ng p53 in Lysis Buffer for 1 h at 4 °C. Beads were washed with

Lysis Buffer 5 x and the bound proteins were analyzed by Western blot.

For binding between Mdm2 and E2, ~100 ng immobilized GST or GST-Mdm2 (unmodified or auto-ubiquitinated) were washed with 50 mM HEPES Buffer and incubated with 1 μg UbcH5c in 50 μl final volume of Lysis Buffer at 4 °C for 2 h. The UbcH5c was either uncharged or thioesterified with ubiquitin prior to incubation with Mdm2. Samples were treated with 15 mM Dithiobis[succinimidyl propionate] (DSP), a thiol-cleavable cross linker (Thermo Scientific), at 22 °C for 2 min. Cross-linking was quenched with 50 mM (final concentration) of Tris-HCl, pH 7.5 for 15 min. After extensive washing, the bead-bound proteins were boiled in sample buffer containing 5% 2-mercaptoethanol to reverse the cross-linking and analyzed by Western blot.

5.2. BRG1 Regulates Mdm2 Stability as a Daxx-HAUSP Binding Partner

Reagents and plasmids

Antibodies against the following proteins/epitopes were obtained from the indicated sources: HAUSP and BRG1 (Bethyl Laboratories); Mdm2 (Ab-1 and Ab-4, Oncogene; SMP-14, Santa Cruz Biotechnology); Flag, HA, and actin (Sigma); p53 (Ab-6, Oncogene); p21 (Cell Signaling); HA and Daxx (Santa Cruz Biotechnology); and GFP (BD Biosciences). MG132, Iodoacetate, anti-FLAG M2 affinity beads, 3x-Flag Peptide, cycloheximide (CHX), and N-ethylmaleimide (NEM) were purchased from Sigma; Protein A/G beads and Lipofectamine 2000 from Invitrogen; anti-HA affinity beads and complete EDTA free protease inhibitors from Roche Applied Science; Glutathione Sepharose 4B beads from GE Healthcare; and senescence detection kit from Biovision.

Flag-BRG1 plasmid was ordered from Addgene (pCMV5 Brg1-Flag; plasmid #19143). pCG/BRM and K798R/pBabe-puro plasmids were obtained from David Reisman at the University of Florida. K798R-Flag was constructed by inserting K798R from pBabe-puro with SalI into pCMV5, then using AgeI and NotI to swap in the N-terminal flag tag from Brg1-Flag/pCMV5. Plasmids encoding HAUSP, Mdm2, p53, and Daxx were previously described (Tang et al 2006). BRG1 truncation mutants were generated by PCR and confirmed by sequencing.

Identification of BRG1 as an interacting protein of HAUSP and Daxx

U2OS cells expressing Flag-HAUSP or Flag-Daxx were cross-linked with 0.2% formaldehyde. The cross-linking reaction was quenched with 0.15 M of glycine (pH7.4). Cell extracts were prepared in the RIPA buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1 mM EDTA, 1% Triton-X-100, 1% sodium deoxycholates, and 0.1 % SDS) supplemented with protease inhibitors, sonicated, and pre-incubated with protein A/G-coupled sepharose beads for 2h at 4 °C. Lysates were then immunoprecipitated with anti-Flag beads for 6h at 4 °C. After beads were extensively washed with RIPA buffer, the bound proteins were eluted from beads using elution buffer (10mM Tris, 100mM NaCl, 2.5mM $MgCl_2$, and 0.4% SDS) at room temperature for 30 min and analyzed by mass spectrometry at the Proteomic Core Facility of the Abramson Cancer Center at the University of Pennsylvania.

Immunoprecipitation and western blot

U2OS cells were treated with MG132 for 6 h before being lysed in the IP lysis buffer (50mM HEPES, pH7.4, 150mM NaCl, 1.5mM $MgCl_2$, 20μM MG132, 10% glycerol, 0.5% NP-40, 0.5% Triton X-100, and protease inhibitors) by gentle sonication. Cell lysates were pre-cleared with protein A/G-coupled Sepharose beads for 2h and immunoprecipitated with the indicated antibodies and isotype-matched control antibodies overnight. Immunoprecipitated proteins and cell lysates were separated by SDS-PAGE followed by western blot.

In vitro binding

Brg1-Flag was expressed in HEK293T cells, treated with MG132 for 6h. and purified with anti-Flag beads as previously described (Tang et al). HA, HA-HAUSP, GST, GST-Daxx, and GST-mdm2 in pRK5 vector were transfected into HEK 293T cells and treated with MG132 for 6h. Lysates were immunoprecipitated with anti-HA or Glutathione beads. Bead bound proteins were first blocked with 3% BSA for 1h before incubating with 100ng purified Flag-BRG1 for 2h at 4°C. Bound proteins were resolved by SDS-PAGE and analyzed by Silver Stain Plus (Bio-rad).

Sequential Immunoprecipitation

HA-HAUSP and GST-Mdm2 were transfected into $p53^{-/-}Mdm2^{-/-}$ MEF cells in the presence or absence of Flag-BRG1. Cells were treated with MG132 for 6 h. Lysates were first immunoprecipitated with anti-Flag beads. Flag-BRG1 and the associated proteins

were eluted with 3xFlag peptide. The eluants were subjected to another immunoprecipitation with anti-Mdm2 or a control antibody followed by Western blot analysis with the indicated antibodies.

Quantitative RT–PCR

Total RNA was isolated from U20S cells by TRIzol Reagent (Invitrogen). Two micrograms of RNA for each sample were reversed to complementary DNA by First-strand cDNA Synthesis System (Marligen Biosciences), and 0.2μg cDNA was used as a template to perform PCR. The primer pairs for human genes were: HDM2, 5'-ATGGTGAGGAGCAGGC-3' AND 5'-CACAGAGAAGCTTGGCA-3'; ACTB, 5′-GACCTGACTGACTACCTCATGAAGAT-3′ and 5′-GTCACACTTCATGATGGAGTTGAAGG-3′

All RT–PCR reactions were performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems) and the amplified using the SYBR Green PCR Master Mix (Applied Biosystems). The thermal cycling conditions were: 50°C for 2min followed by an initial de-naturation step at 95°C for 10min, 45 cycles at 95°C for 15s, 60°C for 1min, and a dissociation curve at 95°C for 15s and 60°C for 15s. The experiments were carried out in triplicate for each data point. Using this method, we obtained the fold changes in gene expression normalized to Actin as an internal control gene.

In vivo ubiquitination assay

BRG1, Mdm2, and p53 were expressed with HA-ubiquitin in $p53^{-/-}Mdm2^{-/-}$ MEF cells. 20 h after transfection, cells were treated with 20μM MG132 for 6 h and then lysed in 1% SDS. After boiling for 5 min, lysates were diluted 10 times with IP lysis buffer

supplemented with 10mM N-ethylmaleimide. Lysates were immunoprecipitated with the indicated antibodies. The immunoprecipitates were subjected to western blot with anti-HA antibody.

In vitro ubiquitination assay

BRG1 (1-650) was cloned with an N-terminal Flag and C-terminal 6x-His tags in the bacterial expression vector pET28a. BRG1 was purified with Nickel beads and eluted before being re-purified with anti-Flag beads. The dual purification scheme was used to ensure we did not obtain truncation or internal initiation products of recombinant BRG1. Ubiquitination reactions consisted of 3-5 ng bead-bound GST-Mdm2, 100 nM E1, 500 nM UbcH5a, -/+ 2 mM Mg^{2+}-ATP, 2 mM DTT, 2-5 μg ubiquitin and 30ng recombinant BRG1 in final volume of 20 μl Ubiquitination Reaction Buffer (40 mM Tris-HCl, pH 7.6). Reaction mixtures were incubated at 37 °C on a microtube orbital shaker (Labnet, Shaker20) at 1,400 rpm and were stopped by addition of sample buffer.

RNA interference

BRG1 siRNA and shRNA were purchased from Santa Cruz and Open Biosystems, respectively. Santa cruz BRG1 SiRNA (it is a mixture) of Sc-29827A (Target sequence: gtacgagtacatcatcaaa), Sc-29827B (Target sequence: ctgctgttctgccaaatga), Sc-29827C (Target sequence: ccgtcaaagtgaagatcaa). Brg1 shRNA from David Reisman target sequences: shRNA-1: CCATATTTATACAGCAGAGAA, shRNA-4: CCGAGGTCTGATAGTGAAGAA. For siRNA transfection, Lipofectamine 2000 (Invitrogen)

was used following the specifications by the manufacturer. To generate lentiviruses expressing BRG1 and control shRNAs, HEK293T cells grown on a 6-cm dish were transfected with 2 μg of pREV, 2 μg of pGag/Pol/PRE, 1 μg of pVSVG, and 2 μg of either BRG1 shRNAs (cloned in PLKO.1) or control vector. 24h after transfection, cells were cultured with DMEM medium containing 20% FBS for an additional 24h. The culture medium containing lentiviral particles were used to infect cells in suspension supplemented with polybrene. Fresh media was added the day after infection.

Cell senescence assay

Senescence assay was conducted using the senescence detection kit from Biovision. Briefly, IMR90 cells expressing BRG1 or control shRNAs were fixed by fixative solution for 15 min at room temperature. After washing twice with PBS, cells were stained with 0.1% X-gal solution for 48 h at 37 °C. The X-gal stained cells were counted under microscope.

Colony formation in soft agar

U2OS expressing BRG1 or control shRNAs were suspended in DMEM containing 10% FBS and 0.3% Seaplaque low melting temperature agarose (Lonza, USA). 1.5 ml agarose containing 1 x 10^4 cells were plated in one well of 6-well plates over a 1.5 ml layer of DMEM/10% FBS/0.6% agarose. Cells were incubated at 37 °C for 3 weeks. The colonies were stained with trypan blue and scored under microscope.

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