NOVEL FUNCTIONS OF USP36: REGULATION OF HISTONE 2B AND THE P53-MDM2 PATHWAY THROUGH DEUBIQUITINATION

By

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Table of Contents

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Abbreviations

4s-UDP	4-thio-uridinediphosphate
aa	amino acid
ACTD	Actinomycin D
ASHL1	Absent, Small, or Homeotic-Like1
ATM	Ataxia telangiectasia mutated
ATR	ATM- and RAD3-related
ATXN7L3	Ataxin 7 like 3
Bre1	BREfeldin A sensitivity
ChIP	chromatin immunoprecipitation
COMPASS	complex proteins associated with Set1p
CTD	C-terminal domain
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco modified Eagle's minimal essential medium
DRB	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ligase
FACT	Facilitates Active Chromatin Transcription
GCN5	Histone asetyltransferase, general control of amino acid synthesis protein 5

H2a	Histone 2a
H2aub1	Monoubiquitinated histone 2a
H2b	Histone 2b
H2bub1	Monoubiquitinated histone 2b
HECT	Homologous to the E6-AP Carboxyl Terminus
IDR	Intrinsically disordered region
IFN	Interferon
IgG	Immunoglobulin G
IRF1	interferon regulatory transcription factor 1
JAMM	Jab1/Mov34/Mpr1 Pad1 N-terminal+
MDM2	Mouse double minute 2 homolog
MJD	Machado- Joseph disease
MLL	Mixed lineage leukemia
MNase	micrococcal nuclease
NELF-E	negative elongation factor-E
NoLs	Nucleolar Localizaiton signal
NPM	Nucleophosmin
OTU	Otubain
P-TEFB	Positive Transcription Elongation Factor beta
PEST	proline (P), glutamic acid (E), serine (S), and threonine (T).
RA	Retinoic acid
RING	Really interesting new gene
RNF20	Ring finger 20

RNF40	Ring finger 40
RPs	ribosomal proteins
rRNA	ribosomal RNA
SAGA	Spt-Ada-Gcn5-Acetyltransferase
STAT1	Signal Transducers and Activators of Transcription 1
SUPt5	Supressor of Ty Homologue-5
TCGA	The cancer genome atlas
ub	ubiquitin
UBC	ubiquitin c
UBE2a	Ubiquitin-conjugating Enzyme E2a
UBE2b	Ubiquitin-conjugating Enzyme 2b
UCH	Ubiquitin C terminal hydrolase
USP	Ubiqtuin specific protease
WAC	WW domain containing adaptor with coiled-coiled domain

List of Figures

List of Tables

List of Appendices

Abstract

Evading cellular programs that limit growth and proliferation is required for both tumor formation and maintenance. The work presented in this dissertation uncovers two novel mechanisms through which USP36, a key nucleolar deubiquitinating enzyme (DUB) implicated in ribosome biogenesis, regulates cell growth and proliferation. First, we show that USP36 is an evolutionarily conserved histone 2b deubiquitinase and that it catalyzes the removal of monoubiquitinated histone 2b (H2bub1). We show that through deubiquitination of this histone mark, USP36 can repress the expression of the cell cycle inhibitor p21. Depletion of endogenous USP36 induced the levels of p21 and markedly suppressed cell proliferation. Second, we report USP36 as a novel DUB regulating the p53-MDM2 signaling pathway. We show that USP36 interacts with, deubiquitinates, and stabilizes both p53 and MDM2. Our results demonstrate that USP36 is a *bona fide* DUB for both MDM2 and p53 and imply that it may play a critical role in regulating p53 activity in cells. Our current work builds upon USP36's established role in promoting ribosome biogenesis and cell growth, through stabilization of c-Myc, Nucleophosmin and RNA Polymerase I. Thus, we provide additional rationale for pursuing it as a therapeutic target.

1 Introduction

1.1 Chromatin Structure & Organization

The human genome is composed of approximately 3 billion base pairs (Makałowski 2001). One fundamental problem encountered by eukaryotic cells is how to physically store such a massive amount of material into a finite nuclear space (reviewed in Talbert & Henikoff 2012). In order to achieve this feat, DNA is packaged into compact fibers. The most basic packaging unit is a nucleosome. Nucleosomes are composed of approximately 147 base pairs of DNA wound around a core of 8 histones, in which 2 copies of each histone (H2A, H2B, H3, H4) are present. The negatively charged phosphate backbone of DNA is electrostatically attracted to the positively charged basic histone core, reinforcing a tightly coiled structure (reviewed in Svaren & Horz 1993). A small stretch of DNA and a fifth histone (H1) link adjacent nucleosomes together. This unit is then repeated throughout the entire length of a chromosome. At this level of organization DNA is compacted into an 11-nanometer fiber (reviewed in Zlatanova & Leuba 2003). This string of nucleosomes folds upon itself to form a solenoid like structure known as the 30-nanometer fiber. This fiber undergoes subsequent rounds of folding, until finally reaching its maximum condensed state known as a chromosome that occurs during meiosis or mitosis. In this state, eukaryotic chromosomes are compacted by an estimated 10,000 – 20,000 fold. In general, packaging solves a storage problem, yet limits access to the underlying DNA sequence and therefore restricts processes such as

transcription, replication and repair. During interphase when these programs occur, chromatin takes on a complex state in which fibers of varying thickness can comingle (Tremethick 2007). In some states, chromatin folding and nucleosome packing can physically block access to the underlying DNA. Chemical modifications that decorate histone tails protruding from nucleosomes can alter the properties of chromatin by promoting either an open or closed state. In general, this ability to restrict access to certain parts of the genome and provide access to others is a fundamental mechanism through which cell identities are determined.

1.1.2 Histone modifications.

Many chemical modifications occurring on histones have been identified. The most common of these include methylation, acetylation, phosphorylation and ubiquitination. Acetylation of basic histone residues neutralizes the positive charge and decreases the affinity of DNA toward the nucleosome core. This results in a more relaxed state that is amenable to nucleosome sliding or eviction. Acetylation is mediated by histone acetyl transferases (HATs) and reversed by histone deacetylases (HDACs) (reviewed in Simone & Peserico 2011). Methylation of arginine and lysine within histone tails is mediated by Histone methyltransferases (HMTs). These methyl groups primarily serve as docking sites for the recruitment of other regulators. For example, proteins containing chromodomains interact with methylation groups present on histones.

Histone methylation can be both a repressive mark (H3K27me3) as well as an activating one (H3K4me3). Finally, histones can be phosphorylated. This modification is involved in DNA damage signaling as well as regulating transcriptional dynamics. Many other, less appreciated modifications have also been found on histones, such as sumolyation, carbonylation, formylation, ADP-ribosylation and O-glycosylation. However their functions have not been fully elucidated (reviewed in Bannister & Kouzarides 2011). While ubiquitination of histones is more rare than acetylation or methylation, this is an important modification involved in regulating chromatin dynamics. Both histone 2A (H2a) and histone 2b (H2b) can be monoubiquitinated (Weake & Workman 2008).

1.2 Monoubiquitination of Hisotne 2b

Monoubiquitination of histone 2b (H2bub1) is a highly conserved modification. In yeast, it occurs at lysine 123 (K123) and in humans it is found on lysine 120 (K120) (Thorne et al. 1987; Robzyk et al. 2000). A single ubiquitin is conjugated to H2b through the canonical ubiquitin conjugation pathway. In the first step, ubiquitin is activated by an E1 activating enzyme. This allows for the transfer of ubiquitin to a ubiquitin conjugating enzyme, known as an E2. Rad6 was identified as the critical E2 for H2bub1 in yeast. There are two human orthologs of Rad6, UBE2a and UBE2b, that mediate monoubiquitination of H2b in vitro (Kim et al. 2009). Knockdown of UBE2a alone is sufficient to dramatically decrease global H2bub1 levels. UBE2a is far more abundant than UBE2b and therefore may be the dominant E2 regulating H2bub1. Finally, an E3 ubiquitin

ligase, Bre1 in yeast and the heterodimer RNF20 and RNF40 in humans, transfer ubiquitin to the substrate (J. Kim et al. 2005). Both RNF20 and RNF40 contain RING (Really Interesting New Gene) finger domains. This domain typically acts as a platform to facilitate direct transfer of ubiquitin from the E2 to the substrate. UBE2a appears to have non-canonical interactions with its E3s in that it does not interact with the RING domains. In spite of this, the RING domain within RNF20 is still required for mono-ubiquitination of H2b, while the same domain is dispensable in RNF40 (Kim et al. 2009). However an interaction between both RNF20 and RNF40 is required for UBE2A binding.

Monoubiquitination of H2b is a chromatin mark associated with active transcription (Minsky et al. 2008). Additionally, it has been shown to directly regulate transcriptional processes. In particular it is known to enhance RNA polymerase II elongation, which in turn, facilitates both transcription and RNA splicing (Zhang et al. 2013; Pavri et al. 2006; Fuchs et al. 2014). Paradoxically, it has also been implicated in negatively regulating gene expression at certain targets (Shema et al. 2011). While this is not entirely understood, there is evidence to suggest that its localization within the gene body may determine whether it has positive or negative effects on transcription (Schulze et al. 2011).

1.2.1 H2bub1 and Transcription

The first indication that H2bub1 was associated with active transcription came long before we understood its enzymatic regulation. In Tetrahymena, it was

appreciated that this mark was abundant in the actively transcribing macronucleus, as opposed to the relatively silent micronucleus (Nickel et al. 1989). Later studies in yeast showed that H2bub1 was a pre-requisite for H3K4me3, mediated by the COMPASS complex of proteins associated with Set1p (Dover et al. 2002). This example of trans-histone crosstalk between H2bub1 and H3K4me3 is correlated with both elongation and active transcription (Krogan et al. 2003; Wood, Schneider, et al. 2003). Furthermore, in yeast, H2bub1 was found to be relatively absent from silent heterochromatic regions, while being enriched in active euchromatin (Kao et al. 2004). In humans, a small scale study looking at a few constitutively active genes, found that the enzymatic machinery regulating H2bub1 was present at both the promoter and coding regions (Zhu et al. 2005). A broader global approach, coupling H2bub1 chromatin immunoprecipitation (ChIP) with mRNA expression levels revealed a widespread association of monoubiquitinated H2b with highly expressed genes (Minsky et al. 2008).

Numerous independent studies have also established a casual role for this modification in transcription by perturbing components of the H2bub1 machinery and observing the subsequent transcriptional effects (J. Kim et al. 2005; Zhu et al. 2005; Pavri et al. 2006; Kim et al. 2009; Shema-Yaacoby et al. 2013; Shema et al. 2008). For example, overexpression of RNF20 is sufficient to induce p21 expression whereas knockdown has the opposite effect (J. Kim et al. 2005). This suggested that H2bub1 may not be merely correlational but may also play an active role in transcription. Interestingly, global expression analysis found that

roughly 8% of mRNAs showed a significant decrease after knockdown of RNF20, albeit the impact was quite varied (ranging from 2-fold to 18-fold) (Shema et al. 2008). Given, the tight correlation between H2bub1 and actively transcribed genes, it was hypothesized that this experiment would yield far more differentially expressed targets. Several single gene studies suggest that H2bub1 perturbation may be the most dramatic at inducible targets that require rapid production of mRNA (J. Kim et al. 2005; Pavri et al. 2006; Kim et al. 2009; Chipumuro & Henriksen 2012). This may partially explain the modest global effect observed following knockdown of RNF20. Another unexpected finding in the global analysis of RNF20 knockdown was that a subset of targets increased in expression. A subsequent study by the same group showed that loss of RNF20 increased recruitment of the transcription elongation factor, TFIIS (Shema et al. 2011), presumably because H2bub1 interfered with its binding at certain loci. Whether this effect is mediated through an RNF20 target other than H2bub1 remains unknown. Furthermore, it is still unclear if the spatial location of H2bub1 within a gene may influence whether it is activating or repressive. Data from yeast studies, showing that the presence of H2bub1 in promoter regions correlates with gene repression (Batta et al. 2011). In spite of having incomplete knowledge about the inhibitory functions of H2bub1 in transcription, there is a significant amount of information about its positive role within the gene body.

1.2.2 H2bub1 and Elongation

The localization of H2bub1 within gene bodies hinted that its transcriptional effects were most likely related to RNA polymerase II elongation as opposed to transcriptional initiation. Nucleosomes act as a natural barrier to RNA polymerase II passage (Kireeva et al. 2002). As such, chromatin remodelers and histone chaperones modify nucleosomes to regulate the elongation process (Venkatesh & Workman 2015). Using in vitro nucleosomal arrays, it was shown that monoubiquitinated H2b stimulates the histone chaperone Facilitates Active Chromatin Transcription (FACT) (Pavri et al. 2006). In one model of FACT function, it displaces an H2A/H2B heterodimer ahead of RNA polymerase II, resulting in a hexameric nucleosome that allows for efficient passage of RNA polymerase II (Hsieh et al. 2013) (Figure 1-1). The ability of H2bub1 to stimulate FACT is critical in the first rounds of transcription. Excluding RNF20/40 from this in vitro system results in a failure to transcribe early on (Pavri et al. 2006). Eventually, in the presence of FACT and a previously defined set of factors, transcription in subsequent rounds does occur. However, it is dramatically enhanced when RNF20/40 is added back. This type of in vitro system may reveal subtleties that are not apparent when bulk mRNA is analyzed from cells. A recent study employed a novel method, 4sUDRB-seq, to examine genome wide elongation rates and coupled this to H2bub1 analysis (Fuchs et al. 2014). They uncovered a strong positive correlation between monoubiquitination of H2b and transcripts with fast elongation rates. Interestingly, they also showed that elongation rates vary within genes and that this also correlates with local levels of H2bub1. In regions where H2bub1 is low, such as exons, there is an

accumulation of RNA polymerase II, interpreted as either stalled or paused. The variance in intron-exon RNA polymerase II elongation rates may occur to

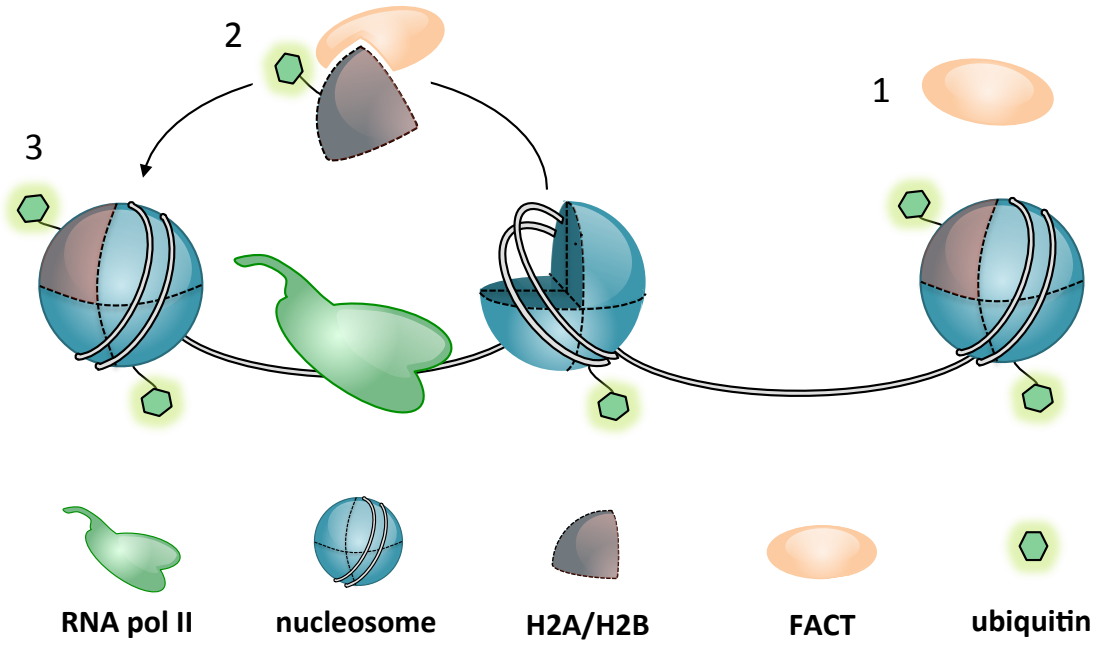

Figure 1-1: H2bub1 facilitates the histone chaperone FACT in RNA polymerase II elongation.

(1) FACT activity is promoted by H2bub1 through an unknown mechanism. (2) FACT displaces H2A/H2B heterodimers altering the way in which DNA is wound around nucleosomes and allows for efficient passage of RNA polymerase II. (3) Following displacement, the H2A/H2B dimers are then reincorporated into a complete octameric nucleosome.

facilitate splicing (Fuchs et al. 2014). Interestingly, several reports indicate that perturbing monoubiquitination of H2b results in 3' splicing defects (Pirngruber, Shchebet et al. 2009, Chipumuro and Henriksen 2012). It is possible that one role for H2bub1 is to fine-tune the elongation rate of RNA polymerase II allowing

for proper co-transcriptional regulated processes such as splicing to occur.

1.2.3 H2bub1 and nucleosome organization

Ubiquitin is a bulky modification compared to other histone PTMs. It has therefore been assumed that its function on H2b would most likely disrupt chromatin structure (Chandrasekharan et al. 2010). Evidence from yeast suggests that unlike acetylation, H2bub1 doesn't destabilize single nucleosomes (Batta et al. 2011; Lee et al. 2012). In fact, it may either stabilize or promote nucleosome reassembly in the wake of transcription. However, H2bub1 does interfere with higher order chromatin structure (Fierz et al. 2011). Recent modeling of the 30nm chromatin fiber indicates that the location of ubiquitin on

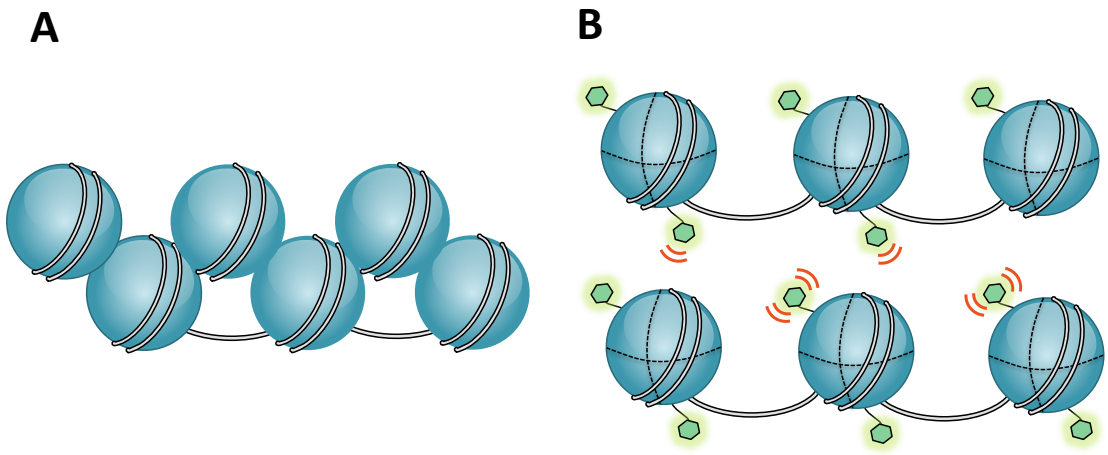

Figure 1-2: H2bub1 alters higher order chromatin structure.

The addition of ubiquitin to H2b results in chromatin decompaction due to an interference with nucleosome stacking. This "open" state gives chromatin-modifying enzymes more access.

H2b interferes with nucleosome stacking (Figure 1-2). Furthermore, an assay utilizing chemically modified H2bub1 to create nucleosomal arrays showed that compaction of the 30nm fiber was significantly inhibited in the presence of H2bub1. Interesting, the yeast protein, HUB1, with an almost identical fold to ubiquitin did not show similar properties. The ability of H2bub1 to open chromatin allows other proteins, such as the methyltransferase DOTL1, that mediates methylation of H3K79, to gain access to nucleosomes (Fierz et al. 2011). Whether this property of H2bub1 modified chromatin underlies its cooperation with other proteins, like FACT, will be interesting to determine.

1.2.4 Histone cross-talk mediated by H2bub1

In addition to regulating elongation and altering higher order chromatin structure, H2bub1 has also been implicated in trans-histone cross talk. This is a process in which a modification on one histone regulates a modification on a different histone. In yeast, this cross-talk pathway between H2bub1 and H3K4 methylation is particularly strong. Deletion of Rad6 from yeast completely abolishes H3K4 di- and tri- methylation mediated by the Set1/COMPASS complex (Dover et al. 2002). Mechanistic studies revealed that the yeast Set1 complex most likely does not interact with H2bub1. Therefore, its role in promoting Set1 activity is not through recruitment. Consistently, in Δrad6 yeast, Set1 is still recruited to chromatin (Kim et al. 2013; Ng et al. 2003). Similarly in humans, DOT1L, a methyltransferase regulating H3K79 methylation that is also

thought to be involved in H2bub1 cross-talk, did not show increased affinity for nucleosomes modified by H2bub1 (McGinty, Kim et al. 2008). Instead, the conformational change to chromatin induced by H2bub1 provides DOT1L with access to nucleosomes (Kim et al. 2013).

In humans the trans-histone crosstalk pathway involving H2bub1 is markedly different. First, there are six methyltransferase complexes that mediate H3K4 methylation, as opposed to one in yeast. There are two set1 orthologs and four mixed lineage leukemia (MLL) family members (Ruthenburg et al. 2007). Second, unlike the situation in yeast, H2bub1 is not a prerequisite for all H3K4 methylations. In contrast, it appears to be important at specific loci regulated by distinct methyltransferase complexes. Of the six human methyltransferase complexes, MLL1 and possibly MLL2 are involved in H2bub1/H3K4me cross-talk (Wu et al. 2013). Interestingly, in contrast to the previously discussed methyltransferases, the MLL1 subunit, ASH2L, has an increased affinity for ubiquitin, which presumably alters the conformation of MLL1 allowing it to mediate methylation at H3K4 (Wu et al. 2013).

1.2.5 H2bub1 occurs co-transcriptionally

Unlike more stable histone modifications such as H3K4me3, H2bub1 is a dynamic modification. Upon induction of p21 by the transcription factor p53, monoubiquitination of H2b rapidly increases and kinetically tracks with RNA polymerase II occupancy in the transcribed regions of the gene. Upon removal of

active p53 from the system, H2bub1 immediately begins to decrease (Minsky et al. 2008). Similar dynamics were noted at the Interferon Regulatory transcription Factor 1 (IRF1) gene, a Signal Transducers and Activators of Transcription 1 (STAT1) target that is induced in response to Interferon gamma (IFNγ) (Buro et al. 2010). Here, IRF1 expression peaks at 90 minutes post IFNγ treatment and then returns close to baseline by five hours. H2bub1 however hits its maximum level at 30 min and then proceeds to decrease.

The dynamic nature of H2bub1 and its tracking with RNA polymerase II occupancy suggest that the two are coupled. Several recent studies have recapitulated an earlier finding that monoubiquitination of histone 2b is dependent on active transcription (Pirngruber et al. 2009; Fuchs et al. 2014) (Pirngruber, Shchebet et al. 2009, Fuchs, Hollander et al. 2014). Treating cells with transcriptional inhibitors such as 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and Actinomycin D (ACTD) results in a rapid decrease of global H2bub1 levels that are quickly recovered upon washout, suggesting that the modification is regulated co-transcriptionally (Davies & Lindsey 1994). Investigating this relationship, Pirngruber et.al found that H2bub1 levels are specifically dependent on CDK9, a subunit of Positive Transcription Elongation Factor beta (P-TEFb) (Pirngruber et al. 2009). CDK9 promotes polymerase II elongation by phosphorylating Suppressor of Ty Homologue-5 (SUPT5) and Negative Elongation Factor E (NELF-E), negative regulators of elongation as well as ser2 in the C-terminal domain (CTD) of RNA polymerase II. However, H2bub1 is not dependent on transcription per se but

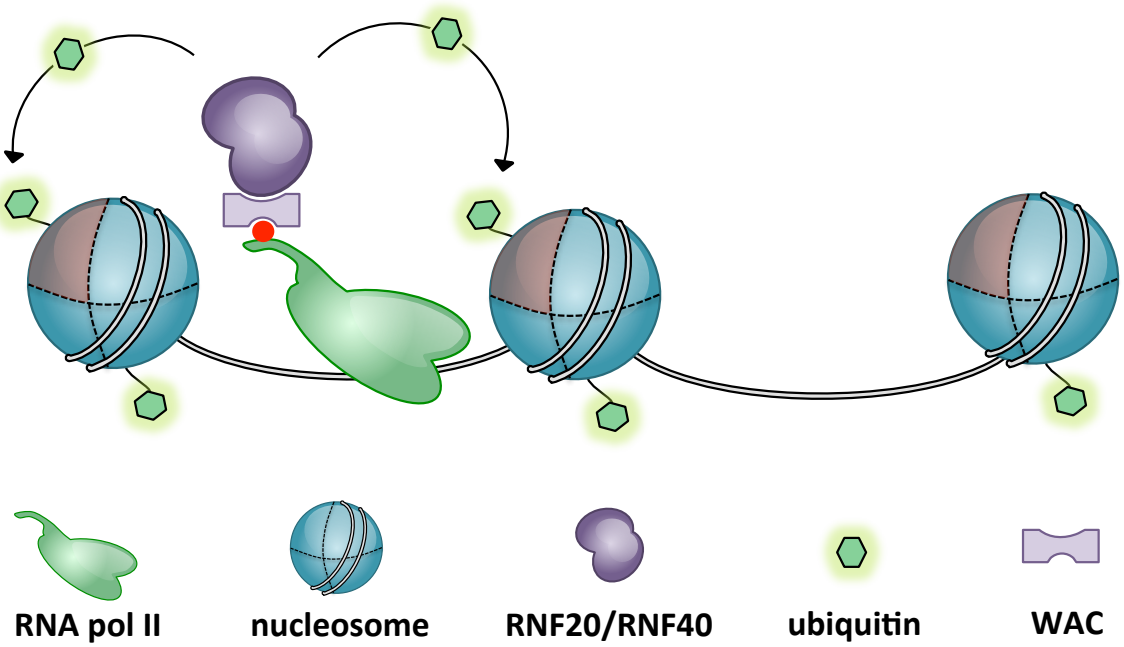

Figure 1-3: H2bub1 occurs co-transcriptionally.

Following phosphorylation of the CTD of RNA polymerase II by CDK9, the adaptc protein WAC binds to phosphorylated ser2. This allows for the recruitment c RNF20/40 which monoubiquitinates H2bub1 either ahead or behind RN/ polymerase II.

specifically on CDK9 mediated phosphorylation of the RNA polymerase II CTD. Following phosphorylation at ser2, WW domain-containing adaptor with coiled-coil (WAC) is recruited to the CTD. RNF20/40 then interacts with WAC through its coiled-coil domain and finally, the E2, UBE2A is brought through RNF20/40 (Figure 1-3) (Zhang & Yu 2011).

1.3 Ubiquitin signaling

Ubiquitin (Ub) is a highly conserved signaling molecule that is 76 amino acids in length and ~8.5KD (Catic & Ploegh 2005). It is covalently linked to the lysines of target proteins or other ubiquitins via its C-terminus. Broadly speaking, ubiquitin is conjugated to substrates as either a polymer or a monomer (Komander et al. 2009). Polymeric chains can be linked through various lysines within ubiquitn (ex: K48, K63, K29). Furthermore, chains can be composed of homogenous linkages or mixed in nature. It is thought that the lysine linkages that occur within chains convey a signaling message (Behrends & Harper 2011). For example, K48 dominant chains primary mediate proteasome-targeted degradation. Whereas, K63 chains regulate endocytosis, cell-signaling and DNA damage (Komander & Rape 2012). As a monomer, ubiquitin is thought to effect function rather than stability. In all cases, the process through which ubiquitin is conjugated is the same.

1.3.1 Ubiquitn conjugation pathway

Ubiquitination is a highly ordered step-wise process that depends on three classes of enzymes: a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3) (Figure 1-4) (Haas & Siepmann 1997). The first step of activation requires an E1 that utilizes ATP to catalyze the formation of ubiquitin adenylate (Schulman & Harper 2009). This charged

intermediate then reacts to form a high-energy thioester bond with the catalytic cysteine in the E1. Ubiquitin is then transferred from the E1 to the active cysteine on an E2, again through the formation of a thioester bond. In the final step, the C-terminus of ubiquitin forms an isopeptide bond with an ε-amino group of a lysine residue either in the target protein or in another ubiquitin molecule, which is mediated by an E3 (Metzger et al. 2014).

The process of ubiquitin conjugation is hierarchically organized, such that the number of enzymes encoded in the human genome for each step successively increases. For example, there are two E1s (UBE1 and UBA6) (Handley et al. 1991; Jin et al. 2007), at least 38 E2s, and more than 650 distinct

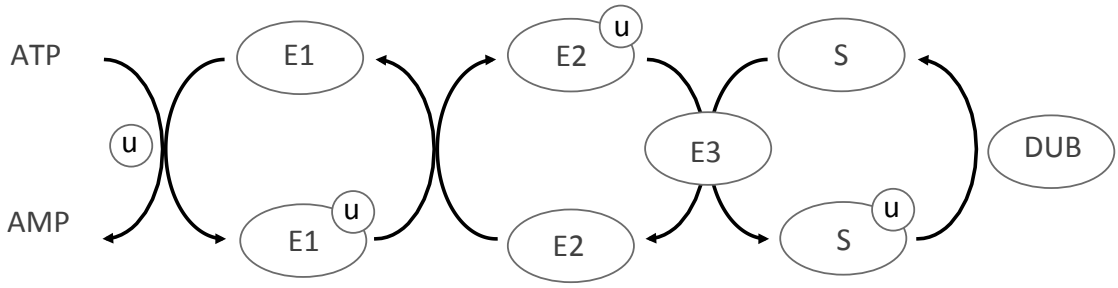

Figure 1-4: Ubiquitin conjugation.

In the first step the E1 ubiquitin activating enzyme is charged with ubiquitin in an ATP dependent manner. The E1 then transfers ubiquitin to an E2 and finally an E3 conjugating enzyme that is specifies the substrate (S) facilitates the final transfer of ubiquitin. DUBs can then reverse the last step.

E3s, each of which has the potential to recognize multiple substrates. Expression of E1s may vary based on tissue type such that each individual cell likely relies

on a single E1 to charge all ubiquitin (Handley et al. 1991; Jin et al. 2007; Ye & Rape 2009). Thus, E1s act on a global level. All E2s share a ubiquitin conjugating (Ubc) domain of approximately 150 amino acids (Wenzel et al. 2011). Given the discrepancy in numbers between E1s, E2s and E3s, any given E2 must interact with both an E1 and numerous E3s. Structural studies show that E1s and E3s share overlap in E2 binding interfaces, indicating that E1–E2 and E2–E3 interactions are most likely mutually exclusive (Eletr et al. 2005).

The E3s represent the largest class of enzymes in the ubiquitin-signaling system, with over 650 human E3s identified. Ubiquitin ligases have been classically split into two groups based on their general mode of catalysis. The HECT (homologous to E6-AP carboxy terminus) domain containing E3s have an identifiable catalytic site, whereas the RING (Really interesting new gene) domain-containing E3s mediate Ub transfer by positioning E2s in close proximity to targets. Canonically, E2s interact with the RING domains, which are essentially platforms for protein-protein interactions (Lorick et al. 1999). Not all proteins that contain RING domains have intrinsic E3 ligase activity. For example, the RING domain of RNF40 is dispensable because it forms a heterodimer with RNF20 in order to carry out ubiquitin transfer (J. Kim et al. 2005).

1.3.2 Deubiquitination

Like other posttranslational modifications, ubiquitination is a reversible process. An entire family of proteins called deubiquitinating enzymes (DUBs) is dedicated to regulating this process. There are approximately 95 DUBs in the human genome that are grouped into five families based on homology within the catalytic domain: the ubiquitin-specific proteases (USPs), the ovarian tumor proteases (OTUs), the ubiquitin C-terminal hydrolases (UCHs), Machado–Joseph disease (MJD) protease, and JAB1/MPN/Mov34 metalloenzymes (JAMMs) (Nijman et al. 2005; Komander et al. 2009). With the exception of JAMMs, which are metalloenzymes, DUBs are cysteine proteases relying on a canonical catalytic triad for their activity. Aspartate polarizes a histidine, which then allows for deprotonation of the active cysteine. Finally, cysteine mediates nucleophilic attack on isopeptide bond, releasing the target protein (Komander et al. 2009).

1.4 Regulation of H2bub1 by Deubiquitination

Monoubiquitination of histone 2b is a modificaiton managed by deubiquitinating enzymes (DUBs) (Frappier & Verrijzer 2011). Like the proteins that regulate ubiquitin conjugation of H2b, the DUBs that mediate its removal are highly conserved (Chandrasekharan et al. 2010). In yeast two DUBs have been identified that regulate H2bub1, ubp10 and ubp8 (Henry et al. 2003; Gardner et al. 2005). Orthologs for both have been discovered in drosophila (Weake et al. 2008; Buszczak et al. 2009). In humans the picture is far more complicated (Table 1). The number of DUBs regulating monoubiquitination of H2b has been

expanded to at least seven. This is consistent with an overall expansion in Ubiquitin Specific Protease (USP) family members (Reyes-Turcu et al. 2009) In

Yeast	*Drosophoila*	*Human*	*Function*
ubp10	Scrawny		primary repressive, although not exclusively
ubp8	Nonstop	USP22	transcriptional activator, SAGA complex
	USP7		transcriptional silencing
		USP3	Unknown
		USP49	regulates splicing
		USP44	transcriptional activator, stem cell maintenance
		USP15	deubiquitinates free histones
		USP42	transcriptional avtivator, at promoter
		USP12	Unknown, potentially a role in development
		USP46	Unknown, potentially a role in development

Table 1: Published DUBs that regulate H2bub1 (as of September 2015).

Published DUBs regulating H2bub1 in yeast, drosophila and humans are indicates as of September 2015. If known, orthologs are listed in the adjacent columns.

yeast there are 16 USP members, whereas in humans there are more than 50 (Nijman et al. 2005).

Deubiquitinating enzymes are essential in regulating the dynamic nature of H2bub1 (Cole et al. 2014). Both addition and removal of ubiquitin from H2b are required for efficient transcription (Buro et al. 2010; Henry et al. 2003; Chipumuro & Henriksen 2012). This cycling behavior was first noted in yeast where the observation was made that both Bre1 and Ubp8 function as transcriptional

activators at the GAL1 locus (Henry et al. 2003). Upon glucose-mediated induction, there is a rapid increase in H2bub1 levels, followed by accumulation of GAL1 mRNA. Before the message hits peak expression, H2bub1 has already begun to decline and maintains a steady state level above its baseline thereafter. Deletion of Bre1 prevents the initial peak of H2bub1, resulting in decreased mRNA (Henry et al. 2003; Kao et al. 2004). On the other hand, when ubp8 is deleted, H2bub1 levels fail to decline and this also negatively impacts GAL1 mRNA levels (Henry et al. 2003).

As mentioned earlier, a positive role for H2bub1 has been well established and is at least partially linked to its effects on higher order chromatin structure

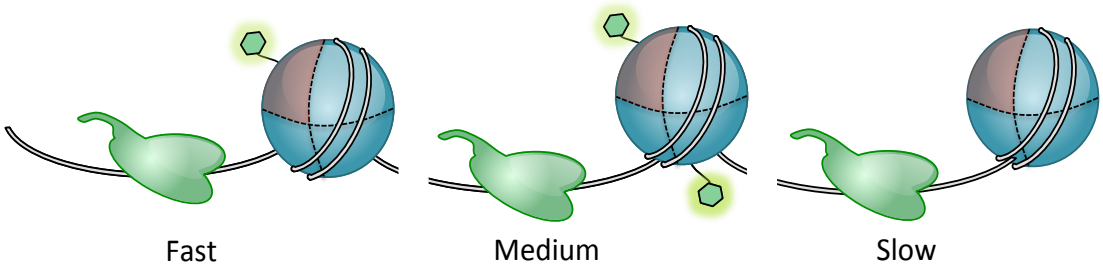

Figure 1-5: A model for RNA polymerase II sensitivity to H2bub1 levels.

When H2b is in its least ubiquitinated state, chromatin may take on a closed conformation in which efficient passage of RNA polymerase II is not favored. However, when both monomers of H2b are ubiquitinated within the octamer, this results in a more open state. It is possible that the bulky H2bub1 lesion may hinder efficient passage of RNA polymerase II. Therefore a state of partial ubiquitination may be the most favorable to polymerase II passage.

(Fierz et al. 2011). When H2b monomers within a histone octamer are maximally ubiquitinated, chromatin takes on a more relaxed state, providing access to factors such as methyltransferases and FACT (Fierz et al. 2011). While the negative effects of H2bub1 are less understood, it is possible that RNA polymerase II is sensitive to the amount of H2bub1 present on a nucleosome (Figure 1-5). For example, a fully ubiquitinated nucleosome, while opening chromatin, may hinder RNA polymerase II passage (Chipumuro & Henriksen 2012). Therefore removal of ubiquitin ahead of RNA polymerase II could facilitate elongation. Whereas co-transcriptional monoubiquitination of H2b mediated by RNF20 may reset nucleosomes in the wake of pol II passage (Figure 1-6**)** (Pirngruber et al. 2009).

1.4.1 Ubp10 and ubp8 in yeast

In yeast two DUBs have been identified that regulate H2bub1 levels, ubp8 and ubp10 (Schulze et al. 2011). Ubp8 is part of the SAGA transcriptional activating complex, which is responsible for its recruitment to chromatin (Henry et al. 2003; Rodríguez-Navarro 2009). Ubp10 is not yet known to form a complex with other proteins. Therefore the mechanism targeting it remains unknown. Deletion of either DUB results in a global increase in H2bub1 (Schulze et al. 2011). They are likely non-redundant because strains in which both are deleted have further increases in H2bub1 beyond a single deletion (Schulze et al. 2011).

Ubp10 was first described as a transcriptional silencer (Gardner et al. 2005). It was shown to interact with silent regions of the yeast genome, such as areas adjacent to telomeres and the HMR mating locus. Mechanistically, ubp10 removes monoubiquitination from histone 2b. This inhibits histone cross-talk with H3K4 and H3K79 methylation and allows Sir4 to gain access to chromatin near telomeres (Gardner et al. 2005). In addition to its role in regulating these silent regions, it also has larger effects on transcription where it appears to function primarily as a repressor NOTCH target genes (Gardner et al. 2005).

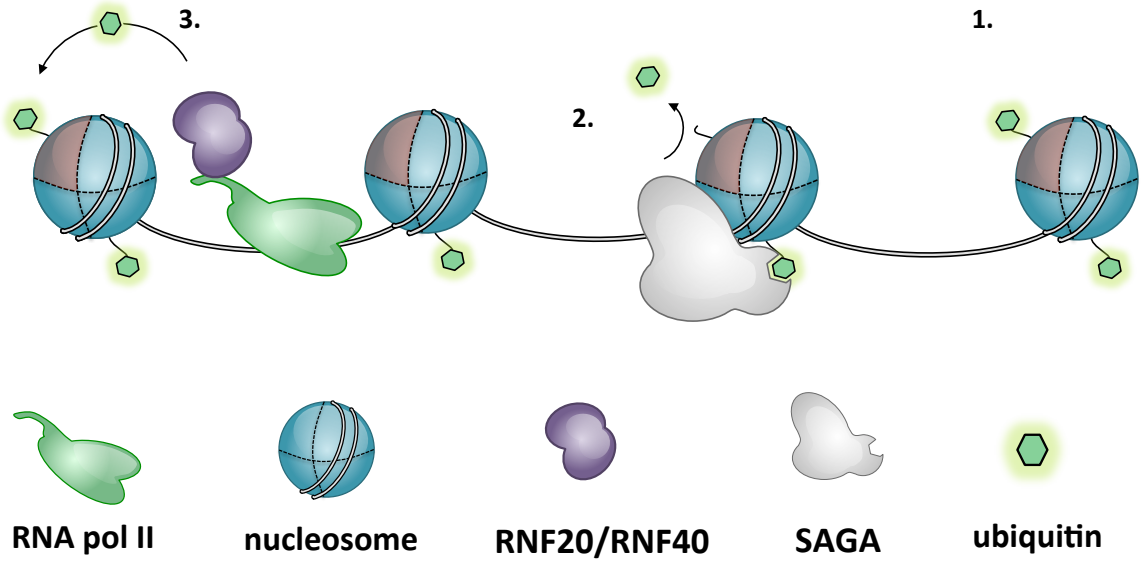

Figure 1-6: Both the addition and removal of H2bub1 positively regulate transcription.

(1) Fully ubiquitinated H2b allows for the recruitment of factors such as SAGA. (2) SAGA is possibly then recruited to chromatin through an interaction with H2bub1. It then removes ubiquitin and this could allow for efficient passage of RNA polymerase II. (3) Finally, RNF20 ubiquitinates H2b in the wake of pol II resetting the open state of chromatin.

Ubp8 as a part of the SAGA complex, on the other hand, is considered a transcriptional activator (Henry et al. 2003). The two DUBs appear to regulate distinct regions of genes. For example, ubp8 deubiquitinates H2bub1 near the transcriptional start sites, whereas ubp10 regulates H2bub1 within the gene body (Schulze et al. 2011).

1.4.2 SAGA, DUBm and USP22

The SAGA complex has two distinct functional modules, one mediating histone acetylation and the other histone deubiquitination. The conserved DUB module (DUBm) contains ATXN7L3, ATXN7, ENY2 and USP22 (Zhao et al. 2008; Zhang et al. 2008; Lang et al. 2011). These four proteins can form a stable complex (Zhao et al. 2008). Interestingly, recombinant USP22 alone has virtually no activity towards H2bub1 present on either free histones or within a nucleosomal array. Furthermore, outside of the DUBm, USP22 is catalytically inactive suggesting that members of the DUBm complex allosterically regulate it (Zhao et al. 2008). Even when USP22 is fully incorporated into DUBm and active, the Zinc Finger (ZnF) domain of ATXN7L3 is still required for deubiquitination of H2b, indicating that ATXN7L3 may reposition DUBm or the nucleosome itself in order for USP22 to gain access to H2bub1 (Zhao et al. 2008). ATXN7L3 is crucial to the deubiquitinating activity of the SAGA complex. Unexpectedly, ATXNL7L3 knockdown has much more dramatic effects than knockdown of

USP22 in terms of global H2bub1 levels (Johnsen 2012). This has led some to speculate whether other DUBs may also function within SAGA.

There are conflicting reports on the actual number of targets regulated by SAGA (Weake et al. 2011; Bonnet et al. 2014). One publication has suggested that SAGA regulates all actively transcribed genes, while others are far more conservative in their estimates (Bonnet et al. 2014). Discrepancies may lie in the fact that different subunits were used for ChIP in the different studies. Future work will resolve these differences. Nevertheless, SAGA is recruited to the promoters of active genes where its histone acetylase (HAT) module regulates H3K9ac (Rodríguez-Navarro 2009). In yeast, it is well established that SAGA also regulates H2bub1 at promoter regions (Schulze et al. 2011). In humans, however, the distribution of H2bub1 is slightly different with heavy enrichment within the gene body (Bonnet et al. 2014). The extent of H2bub1 present within promoters is far lower. However, there are single gene observations indicating that USP22 can regulate H2bub1 at promoters (Zhang et al. 2008; Zhao et al. 2008). In order to resolve this, USP22, specifically as opposed to ATXN7L3 should be knocked down followed by examination of global H2bub1 by ChIP and mRNA profiling.

How SAGA is recruited to chromatin is still somewhat unresolved although there is strong evidence to suggest that H2bub1 itself may be involved (Bonnet et al. 2014). A recent study found that upon ATXN7L3 knockdown, the increase in H2bub1 was proportional the amount of H2bub1 already present. This suggests a model in which SAGA is recruited via one H2b monomer within an octamer to

deubiquitinate the other (Bonnet et al. 2014). This raises the intriguing possibility that other H2bub1 DUBs could then regulate SAGA activity.

1.4.3 USP15

USP15 was identified as an H2bub1 interacting protein in a screen utilizing chemically synthesized monoubiquitinated H2b (Long et al. 2014). USP15 was found to preferentially deubiquitinate free histones as opposed to those incorporated into nucleosomes. Additionally, they found an interaction between USP15 and SART3, another hit in the screen. SART3 is best known for its role in regulating splicing but also functions as a histone chaperone. Like USP15, SART3 has a preference for histones outside of nucleosomes, which is consistent with its chaperone function. Additionally, it enhances the activity of USP15. The authors propose that the two proteins may deubiquitinate H2B that is evicted as RNA polymerase II passes, which is then re-deposited back into a functional nucleosome (Long et al. 2014).

1.4.4 USP44

USP44 was identified as a candidate H2bub1 DUB in a microarray analysis of genes downregulated following retinoic acid (RA) induced differentiation (Fuchs et al. 2012). Upon RA treatment, there is a dramatic increase in the global levels of H2bub1, while RNF20 remains relatively constant.

USP44, a DUB that shares homology with other H2bub1 DUBs, is significantly downregulated. The negative correlation between USP44 and H2bub1, led the authors to test whether it could deubiquitinate H2b. Indeed it was shown that USP44 can alter both global and gene specific H2bub1 levels. Similar to USP22 and USP42, deubiquitination of H2bub1 at 5' transcribed regions appears to result in a transcriptional outcome similar to RNF20 mediated ubiquitination (Fuchs et al. 2012; Chipumuro & Henriksen 2012; Henry et al. 2003). Although seemingly contradictory, this supports the idea that H2bub1 is actively cycled during transcription and furthermore that this process is required for efficient expression of specific targets. Interestingly, changes in other H2bub1 DUBs such as USP3 and USP22 were not observed during differentiation (Fuchs et al. 2012). Furthermore, the dramatic changes in global H2bub1 following perturbation of USP44 in a stem cell model were not seen in similar experiments done with USP22. Altogether, this suggests that USP44 may be the dominant H2bub1 DUB in stem like states, whereas others such as USP22 may be more important in normal cells upon differentiation (Fuchs et al. 2012).

1.4.5 USP49

USP49 is unique in its specificity for H2bub1, whereas most other DUBs have dual specificity towards both H2bub1 and H2aub1 (Zhang et al. 2013). USP49 alone is not sufficient to deubiquitinate H2bub1. Instead it forms an active complex with RVB1 and SUG1. RVB1 is a helicase that is known to interact with

other chromatin remodelers in order to facilitate interactions with nucleosomes. SUG1 is an ATPase that is part of the 19S proteasome regulatory subunit. As a complex, USP49-RVB1-SUG1 is capable of deubiquitinating H2bub1 within the context of nucleosomes. In an effort to determine the global targets regulated by USP49, Zhang et. al carried out gene expression analysis following knockdown of USP49 (Zhang et al. 2013). Surprisingly, they uncovered a dramatic difference in expression of splice isoforms. While it is generally accepted that H2bub1 is highest within the coding regions of genes, several groups have made the observation that it is particularly enriched at intron-exon borders. Upon knockdown of USP49, there is a further increase and persistence of H2bub1 at intron-exon borders. Somehow this hinders the recruitment of splicing factors U2B and U1A to both chromatin and mRNA. As a result, un-spliced transcripts that still contain introns were found enriched on chromatin and lacked the ability to be released. Interestingly, when USP22 and USP12, two other H2bub1 DUBs, were knocked down and tested for splicing defects, none were found. This suggests that the co-transcriptional regulation of splicing by modulating H2bub1 at intron-exon boundaries may be specific to USP49 (Zhang et al. 2013).

1.4.6 USP42

USP42 was recently shown to positively regulate transcription of many p53 target genes, including p21 (Hock et al. 2014). It promotes p53-mediated transcription through two separate mechanisms. Early in activation, USP42

stabilizes p53, which impacts transcription of target genes. However, in later phases USP42 has no effect on p53 stability and yet p53 target gene expression never fully recovers in the absence of USP42. Chromatin immunoprecipitation analysis showed that USP42 is recruited to the p21 promoter in the area adjacent to the transcriptional start site (TSS), where it regulates H2bub1 levels. In the absence of USP42, H2bub1 increases near the TSS, accompanied by increased RNA polymerase II levels that are indicative of stalling. Consistent with its accumulation at the TSS, RNA polymerase II is also reduced within the gene body. Mechanistically, this suggests that H2bub1 may play a negative role at promoters by preventing paused RNA polymerase II from switching to an elongating phase. This study indicates that in spite of the relatively low levels of H2bub1 within the p21 promoter when compared to the gene body, there is still a significant effect on the ability of RNA polymerase II to undergo elongation. Overall, USP42 appears to be unique in its positional regulation of H2bub1 (Hock et al. 2014). Given the failure of RNA polymerase II to travel through the gene body in the absence of USP42, one might predict a decreased levels of H2bub1 within the gene body, however the authors did not test this.

1.4.7 Other DUBS : USP46/USP12 , USP3

Several other DUBs, USP3 and the complex USP12/USP46 can deubiquitinate H2bub1 in vitro, in vivo or both (Joo et al. 2011; Nicassio et al. 2007). In all instances the significance of H2bub1 deubiquitination was not

characterized. Instead these DUBs were studied in the context of deubiquitinating histone 2A. Future work will determine the significance of their activity toward H2bub1.

1.5 USP36: protein

As mentioned, there are two yeast DUBs that mediate deubiquitination of H2bub1; ubp8 and ubp10. While the human ortholog of ubp8 is known (USP22), the ortholog of ubp10 remains unknown. The human DUB, USP36, is the predicted ortholog of ubp10 based on high degree of similarity between the catalytic domains of the two proteins (Figure 1-10). USP36 belongs to the Ubiquitin Specific Protease (USP) family. The gene is located distally on the long arm of chromosome 17 (17q25.3). The full-length transcript is 5234bp and codes for a protein that is 1121 amino acids long (Li et al. 2008). Initially, a shorter N-terminal transcript, named DUB-1 (541aa), was cloned from Hela cells (Kim et al. 2004). In addition to DUB-1, at least five other splice variants exist (Li et al. 2008). However, the expression and function of these transcripts has not been fully assessed and therefore, their significance remains relatively unknown. One report has explored the expression of the full-length transcript and a highly expressed splice variant in normal ovaries vs. ovarian cancer tissue (unmatched) (Li et al. 2008). The full-length transcript was found in all tumor samples, whereas it was absent from normal ovaries. Expression of the two transcripts

was also assessed in normal pancreas, kidney, cervix, spleen, testis, endometrium, breast, colon, myometrium, and liver. In this analysis the full-length

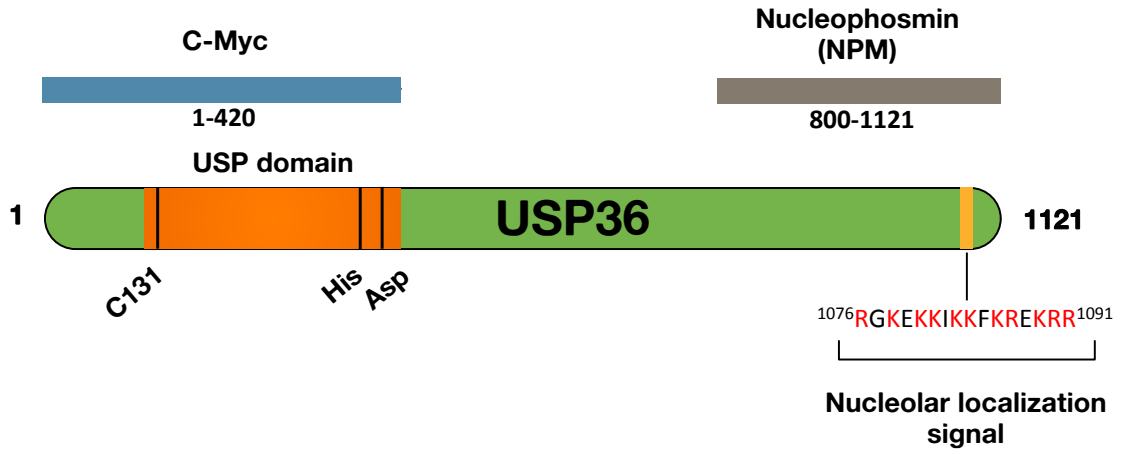

Figure 1-7: Schematic of USP36 protein.

USP36 is 1121 amino acids long. The UCH domain, with 4 conserved subdomains, is located in the N-terminus. The asterisk denotes C131. The NoLS is found in the C-terminus, indicated by a purple bar. Its highly basic sequence is shown. Binding of c-Myc and Nucleophosmin has been roughly mapped and the fragments are indicated.

transcript was absent from all normal tissues, whereas the splice variant was found to be ubiquitously expressed. Interestingly, this shorter transcript is missing the first 300 amino acids and thus the majority of the catalytic domain. However, the C-terminus, which contains the nucleolar localization signal (discussed below), is present. Because the analysis of normal tissue relied on a set of RT-PCR primers within the 3' end of the mRNA, it's not clear if they may have been interrogating an unknown transcript. More work needs to be done to assess the expression of human USP36 and variants in both tumors and normal tissue (Li et al. 2008).

The USP domain of USP36 (122 – 420) contains four highly conserved subdomains that coordinate catalysis; the Cys domain (123-140), Asp (I) domain (201-16), and His domain (367-384) (Figure 1-7) (Endo, Kitamura, et al. 2009). Mutating the key cysteine residue (C131), found within the Cys domain abolishes its catalytic activity (Kim et al. 2004). Outside of the highly conserved catalytic domain, the rest of the protein is unstructured. The intrinsically disordered regions (IDRs) of USP36 may confer conformational plasticity leading to the formation of specific protein interaction sites, similar to the way in which IDRs function in the USP36 yeast ortholog, ubp10 (Reed et al. 2015). Additionally, they may fold/unfold to expose protein cleavage sites. For example, several PEST motifs (a run of at least 12 amino acids enriched in proline P, glutamate E, serine S, and threonine T) have been found within the unstructured region (M. S. Kim et al. 2005). These sequences are primarily associated with rapid degradation of proteins (Spencer et al. 2004).

1.5.1 Nucleolar Specific Functions of USP36

While the disordered secondary structure of USP36 limits predictions that can be made about its function, its C-terminal sequence contains a nucleolar localization signal (NoLS) that provides information about the targets and processes it regulates (Endo, Kitamura, et al. 2009). A C-terminal basic motif

enriched in lysine and arginine found at 1076-1091 is both necessary and sufficient for USP36 localization to the nucleolus (Figure 1-7). Endo et al. showed that the nucleolar protein, Nucleophosmin (NPM), interacts with the basic motif of USP36 through an acidic patch on the protein and directs its nucleolar localization (Endo, Kitamura, et al. 2009). Loss of NPM phenocopies deletion of the basic motif in terms of localization. This suggests that at least in Hela cells, this interaction is a primary driver of USP36 localization. NPM forms a large homo-oligomer complex that interacts with numerous proteins anchoring them in the nucleolus. Interestingly, NPM is also a substrate of USP36. Therefore USP36 stabilization of NPM may promote nucleolar retention of itself and other nucleolar proteins (Endo, Kitamura, et al. 2009). USP36 is most likely involved in numerous nucleolar processes. It is known to stabilize Fibrillarin and NPM, both of which are involved in ribosomal RNA (rRNA) processing (Endo, Kitamura, et al. 2009). Loss of USP36 modestly delays 47S rRNA processing and subsequent steps (Endo, Kitamura, et al. 2009). There is mounting evidence that another nucleolar function of USP36 is to regulate rRNA transcription (Sun et al. 2015; Endo, Kitamura, et al. 2009; Peltonen et al. 2014). In addition to slowed processing of the 47S rRNA, there is also substantially less 47S to start with in USP36 depleted cells. There are two possible targets through which USP36 may regulate rRNA transcription (Endo, Kitamura, et al. 2009). First, our lab has shown that it regulates the stability of c-Myc. rRNA production is known to be stimulated by c-Myc, which recruits TRRAP to increase acetylation of histones and promotes binding of the SL-1 pre-initiation complex that interacts with RNA

polymerase I (Sun et al. 2015; Arabi et al. 2005). A second, more direct route, through which USP36 may regulate rRNA production is by stabilizing the largest RNA polymerase I subunit, RPA194 (Richardson et al. 2012; Peltonen et al. 2014). It was recently shown that overexpression of USP36 can rescue the proteasome mediated degradation of RPA194 induced by the anti-cancer agent BMH-21 (Peltonen et al. 2014). This appears to be a conserved function of USP36 as its ortholog, ubp10, has also been shown to stabilize the analogous subunit of RNA polymerase I in yeast (Richardson et al. 2012).

1.5.2 A Role in Cancer

Several lines of evidence suggest that USP36 may have oncogenic functions. First, an examination of TCGA (The Cancer Genome Atlas) data across a wide spectrum of cancers reveals that USP36 is often overexpressed and/or amplified (Cerami et al. 2012). In contrast there are very few instances of deletion or down regulation (Figure 1-8). Additionally, there are no sites of concentrated mutations. For example, mutations within the catalytic domain are the most likely to result in loss of function and these appear to occur at a similar density with mutations found throughout the protein. This can be contrasted with BAP1, a DUB known to function as a tumor suppressor, in which recurrent mutations predicted to impact function are common within the catalytic domain

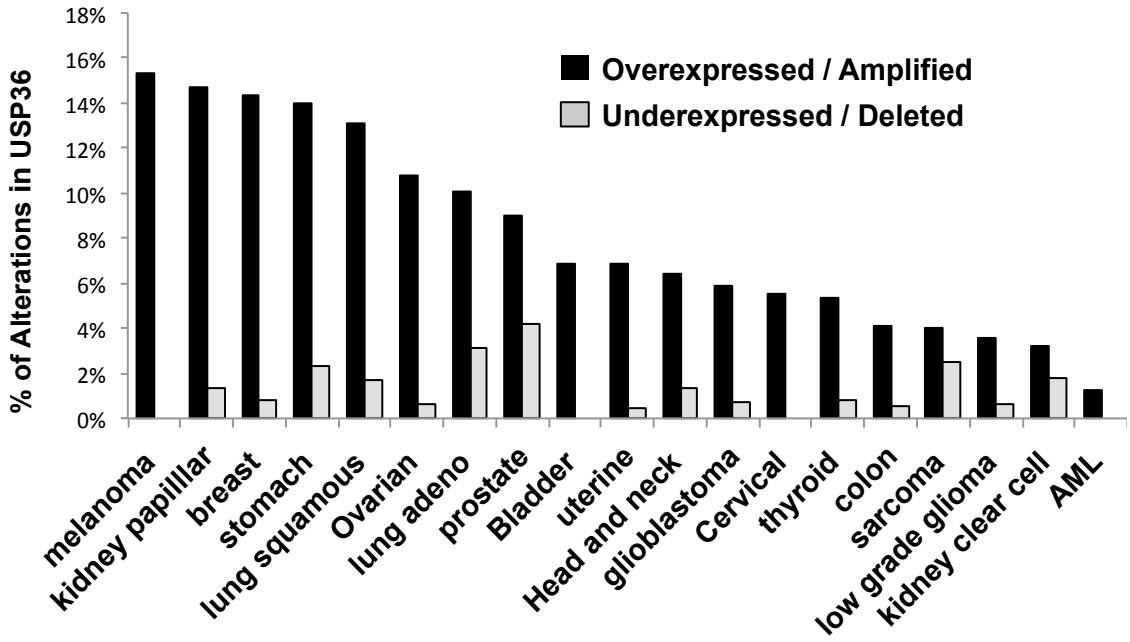

Figure 1-8: USP36 is amplified or overexpressed in cancer.

TCGA data for USP36 was collected from wide a spectrum of cancers using the cBio portal. Overexpression is combined with instances of amplification and indicated by a black bar. Underexpressed or deleted cases are shown in grey. The y-axis is the percentage of patients with alterations in these two categories. http://www.cbioportal.org

(Figure 1-9) (Harbour et al. 2010; Abdel-Rahman et al. 2011; Popova et al. 2013; Testa et al. 2011). Finally, as mentioned, USP36 was identified as a hit in a screen for targets that are differentially expressed in ovarian cancer (Li et al. 2008). Strikingly, in patient derived cell lines, USP36 was almost absent from normal tissue (Li et al. 2008). In support of its oncogenic role in cancer, we and others have found that knocking down USP36 results in decreased viability (Sun et al. 2015; Endo, Matsumoto, et al. 2009). This observation has been made in all cell lines we have tested. Interestingly, Endo et al. found that the structure of

the nucleolus was partially disrupted when USP36 was knocked down (Endo, Matsumoto, et al. 2009). It is known that the reassembly of nucleoli after cell division is dependent on RNA polymerase I transcription

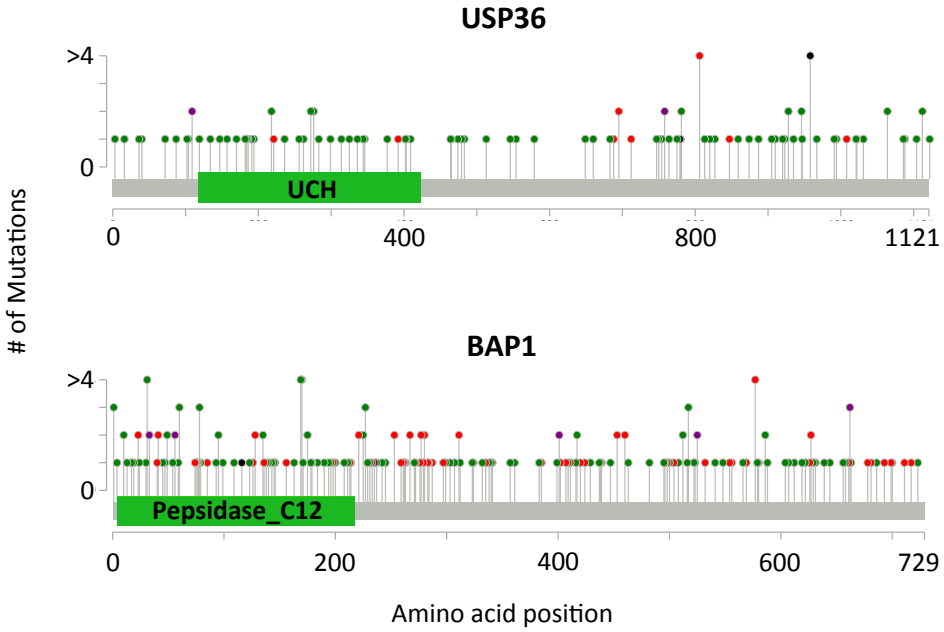

Figure 1-9: Recurrent mutations within USP36 are not common in cancer. Mutation profiles for both USP36 and BAP1 taken from all cancer types using cBio portal.

(Hernandez-Verdun 2006). In this context, they speculate that decreased rRNA transcription in USP36 depleted cells may result in nucleolar reassembly defects (Endo, Matsumoto, et al. 2009). Additionally, destabilization of the master regulator, c-Myc, is another mechanism through which loss of USP36 negatively impacts cancer cell proliferation (Sun et al. 2015). Surprisingly, overexpression of c-Myc in the background of depleted USP36 further decreased cell viability

indicating that USP36 has a wide range of effects that are intimately tied to viability (Dai observation).

1.5.1 Orthologs and Conserved Functions

Two orthologs of USP36 have been identified and characterized in yeast (ubp10) and drosophila (dUSP36/Scrawny) (Reed et al. 2015; Richardson et al. 2012; Buszczak et al. 2009). Figure 1-10 shows the high degree of similarity (>50%) between the structured protease domain of USP36 and its two orthologs. As mentioned above, the majority of sequence flanking the catalytic domain is highly unstructured (Reed et al. 2015). This is also the case for both ubp10 and dUSP36. In general both orthologs have a diverse target repertoire and regulate numerous processes (Gardner et al. 2005; Schulze et al. 2011). For example, ubp10 is involved in telomere silencing, regulating gene expression, the DNA damage response, and stabilizing RNA polymerase I (Gardner et al. 2005; Schulze et al. 2011; Reed et al. 2015; Cao & Yan 2012). dUPS36 has also been identified as a transcriptional repressor of NOTCH target genes (Buszczak et al. 2009). There is strong evidence that points to USP36 as a functional ortholog of both ubp10 and scrawny. Like dUSP36 in drosophila, knockdown of USP36 induces autophagy in human cells (Taillebourg et al. 2012). In yeast, rescue experiments have been carried out, in which human USP36 can compensate for loss of yeast ubp10. The Gardener has group showed that upon deletion of ubp10, there is a dramatic decrease in RPA190 stability (Richardson et al. 2012).

This can be fully rescued by expressing wild type human USP36, whereas the catalytic mutant fails to rescue RPA190 stability. Following this finding, it was

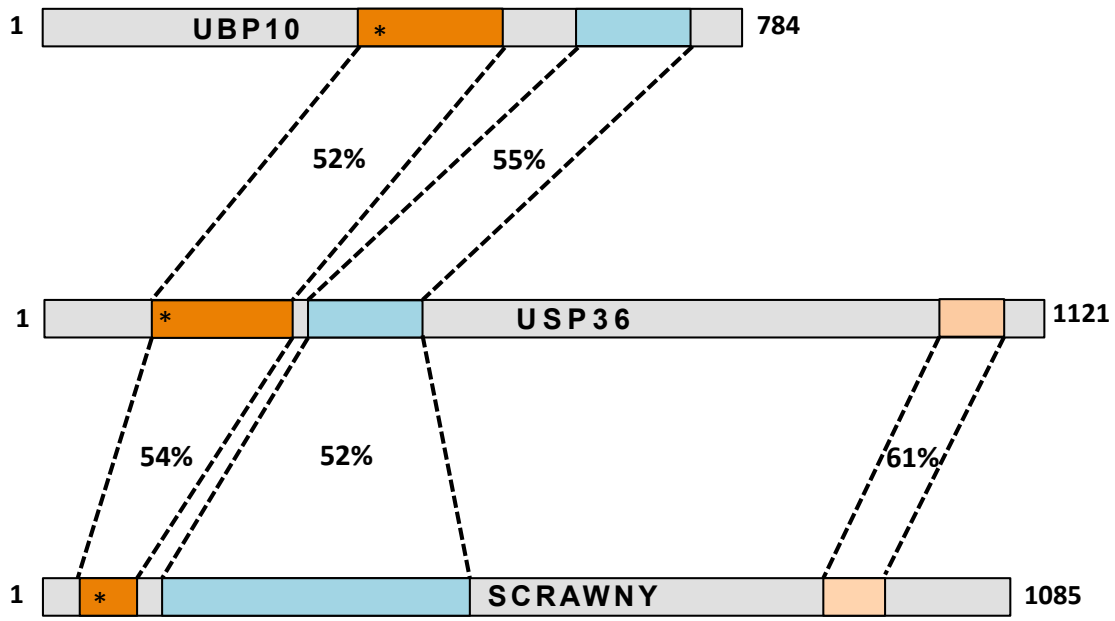

Figure 1-10: Orthologs of USP36.

Scrawny is the drosophila ortholog of USP36. Ubp10 is the yeast ortholog. whereas ordered regions are a light grey line. The regions of matching color indicate where similarity analysis occurred. The % similarity is then shown between USP36 and the ortholog and corresponds to the color-matched region. http://hmmer.janelia.org/

later shown that USP36 stabilizes an analogous RNA polymerase I subunit (RPA194) in human cells (Peltonen et al. 2014). Additionally, a study in yeast strongly suggests that USP36 conserves the ability to regulate H2bub1. When ubp10 is deleted, this results in globally increased H2bub1. Concomitant

overexpression of wild-type USP36 and not the catalytically inactive mutant partially rescues H2bub1 levels.

The similarity between the catalytic domains of human USP36, ubp10 (yeast) and Scrawny (drosophila) as well as their overlap in function led us to postulate that like ubp10 and Scrawny, USP36 may conserve the ability to deubiquitinate monoubiquitinated histone 2b. Furthermore, it is known that global H2bub1 levels decrease with cancer progression suggesting that the enzymes regulating this modification may be deregulated in cancer (Prenzel et al. 2011; Du et al. 2014; Z. J. Wang et al. 2013; Wang et al. 2015; Johnsen 2012). This is consistent with the observation that USP36 is overexpressed in cancer (Figure 1-8). Furthermore, we hypothesize that deubiquitination of histone 2b supports the oncogenic functions of USP36.

2 USP36 is a conserved H2bub1 Deubiquitinating Enzyme that Represses p21 Expression

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2.1 ABSTRACT

Histone H2b monoubiquitination (H2bub1) plays a critical role in the regulation of gene transcription. Increasing evidence also suggests that H2bub1 is negatively correlated with the progression of malignancies, such as breast and gastric cancers. RNF20, an enzyme mediating H2b monoubiquitination, and USP22, an enzyme deubiquitinating H2bub1, have been implicated as a tumor suppressor and an oncogene, respectively. Here we report that human USP36 is a novel H2bub1 deubiquitinase, conserving the function of its yeast and drosophila orthologs, ubp10 and Scrawny. We show that USP36 interacts with H2b and deubiquitinates H2bub1 in cells and in vitro. Overexpression of USP36 drastically reduced the levels of H2bub1 in cells. Using the p21 gene as a model, we demonstrate that USP36 mediates transcriptional repression at the p21 locus by deubiquitinating H2bub1 primarily within its gene body. Consistently, knockdown of USP36 significantly induced the levels of p21, resulting in the inhibition of cell proliferation and induction of cell senescence. Together, our results shed light on additional oncogenic functions of USP36 and further supports rationale to targeting USP36 in cancer therapy.

2.2 INTRODUCTION

Posttranslational modifications of core histones play a key role in regulating chromatin dynamics, gene transcription, and DNA repair (Bannister & Kouzarides 2011b). Monoubiquitination of Histone 2b is an evolutionally conserved modification. A single ubiquitin E3, Rad6/Bre1, mediates H2b monoubiquitination at Lys 123 (K123) in yeast whereas the orthologs RNF20 and RNF40 form a complex to primarily mediate H2b monoubiquitination at K120 in human (Hwang et al. 2003; J. Kim et al. 2005; Thorne et al. 1987). Monoubiquitinated H2b (H2bub1) is generally enriched at actively transcribed genes and positively correlated with their expression levels (Kao et al. 2004; Minsky et al. 2008; Zhu et al. 2005). It has been implicated in transcription elongation and co-transcriptional pre-mRNA processing, as well as histone cross-talk (Dover et al. 2002; Krogan et al. 2003; Wood, Krogan, et al. 2003). H2bub1 stimulates the function of the histone chaperone FACT and promotes efficient RNA polymerase II passage during transcription elongation (Pavri et al. 2006). Thus, H2b monoubiquitination plays an important role in regulating gene expression. Not surprisingly, deregulation of this modification contributes to human pathologies including cancer (Prenzel et al. 2011; Du et al. 2014; Z. J. Wang et al. 2013; Wang et al. 2015; Johnsen 2012). In support of this notion, several studies have found a negative correlation between H2bub1 levels and tumor progression. Consistently, the E3 ligase RNF20 is lost through hypermethylation of its promoter in breast cancer and this correlates with

decreased H2bub1 levels in more progressed states, indicating that RNF20 may be an important tumor suppressor (Prenzel et al. 2011).

On the other hand, H2bub1 can be reversed by deubiquitination catalyzed by deubiquitinating enzymes (DUBs). While H2bub1 is primarily mediated by a single E3 in both yeast and humans, there appear to multiple enzymes regulating its removal. In yeast, two DUBs, ubp8 and ubp10, deubiquitinate H2bub1 (Henry et al. 2003; Gardner et al. 2005). Ubp8 (USP22 in humans) is a subunit of the DUB module within the SAGA (Spt-Ada-Gcn5-acetyltransferase) complex that may broadly be required for transcription (Henry et al. 2003, Bonnet et al. 2014). Consistently, USP22 has been identified as a key oncogene, where its presence predicts lower overall survival in numerous cancers (Glinsky 2005; Li et al. 2013; Y.-L. Liu et al. 2011; Lv et al. 2011; Piao et al. 2012; Piao et al. 2013; H. Wang et al. 2013; Yang et al. 2013; Y. Zhang et al. 2011; Yang et al. 2011). In contrast, ubp10 has been shown to act as a transcriptional repressor (Gardner et al. 2005). It associates with the silent regions of the yeast genome, including areas adjacent to telomeres and the HMR (Hidden Mat Right) mating locus (Gardner et al. 2005). In addition, ubp8 and ubp10 appear to regulate distinct regions of genes, with ubp8 deubiquitinating H2b near the transcriptional start sites and ubp10 deubiquitinating H2b within the gene body (Schulze et al. 2011). Similar functions have been reported for drosophila DUBs, nonstop and Scrawny, the ubp8 and ubp10 orthologs, respectively (Weake et al. 2008; Buszczak et al. 2009). Like ubp10, Scrawny has also been described as transcriptional repressor (Buszczak et al. 2009).

In humans, a handful of DUBs have been shown to catalyze the removal H2bub1 in addition to USP22, including USP44, USP42, USP49, USP3, and USP15 (Chipumuro & Henriksen 2012; Zhang et al. 2008; Zhao et al. 2008; Fuchs et al. 2012; Nicassio et al. 2007; Long et al. 2014). This increase in enzymatic complexity is most likely indicative of a wider breadth of regulatory functions for H2bub1. Indeed, three of the DUBS, USP22, USP44, and USP42, are involved in positively regulating transcription, albeit through distinct target sets (Hock et al. 2014; Fuchs et al. 2012; Chipumuro & Henriksen 2012). Currently no human DUBs have been described that like ubp10 in yeast repress transcription through the removal of H2bub1. While ubp10 is known to mediate gene silencing, its H2bub1 mediated transcription effects are more complicated and not strictly limited to suppression. It does however associate with silent regions of the genome and is known to regulate repression of subtelomeric loci (Gardner et al. 2005). Importantly, deletion of ubp10 results in global mRNA expression changes that are linked to loss of H2bub1 (Gardner et al. 2005; Schulze et al. 2011). Yet, whether the putative ubp10 ortholog, human USP36, can regulate H2bub1 and transcription in human cells is unknown.

In this study, we show that human USP36 conserves the function of its yeast and drosophila orthologs Ubp10 and Scrawny, respectively to deubiquitinate H2b. Overexpression of USP36 markedly increased the global H2bub1 levels in cells. Using the model gene CDKN1A (coding for p21), we demonstrate that USP36 can interact with the p21 gene body and deubiquitinate H2bub1 within this region. Consistently, we show that knockdown of USP36

increased p21 levels, leading to inhibition of cell proliferation and the induction of senescence. Together, these results suggest that USP36 is an important regulator of H2bub1 and functionally consistent with its role in cell growth and proliferation.

2.3 RESULTS

USP36 deubiquitinates mono-ubiquitinated histone 2b. To determine USP36 has a conserved function in deubiquitinating histone 2b, we examined its effect on global H2bub1 using a monoclonal antibody that specifically recognizes the branch site where ubiquitn is conjugated to histone 2b (Minsky et al. 2008). As shown in Figure 2-1A, H2bub1 levels markedly decreased upon overexpression of USP36 in a dose-dependent manner in H1299 cells, while the levels of total H2b remained unchanged. To determine whether the decreased H2bub1 was dependent on the catalytic activity of USP36, we utilized a catalytic-inactive C131A point mutant of USP36 ($USP36^{C131A}$) (Endo, Kitamura, et al. 2009). Unlike wild-type USP36, the catalytic mutant did not decrease H2bub1 levels (Figure 2-1B). Similar results were also observed in other tested cell lines. Next we examined whether USP36 could directly deubiquitinate H2bub1 by performing an *in vitro* DUB assay. Histones purified from H1299 cells by acidic extraction were incubated with either a His-tagged wild-type recombinant USP36 N-terminal fragment (His-$USP36^{1-800}$) or the catalytically inactive mutant (His-$USP36^{1-800\ C131A}$). As shown in Figure 2-1C, only wild type USP36 was capable of deubiquitinating H2bub1 *in vitro*. Together, these data demonstrate that USP36 is indeed a bona fide DUB for H2bub1.

USP36 interacts with histone 2b. We next sought to examine how USP36 deubiquitinates H2bub1. To this end, we tested whether it was capable of

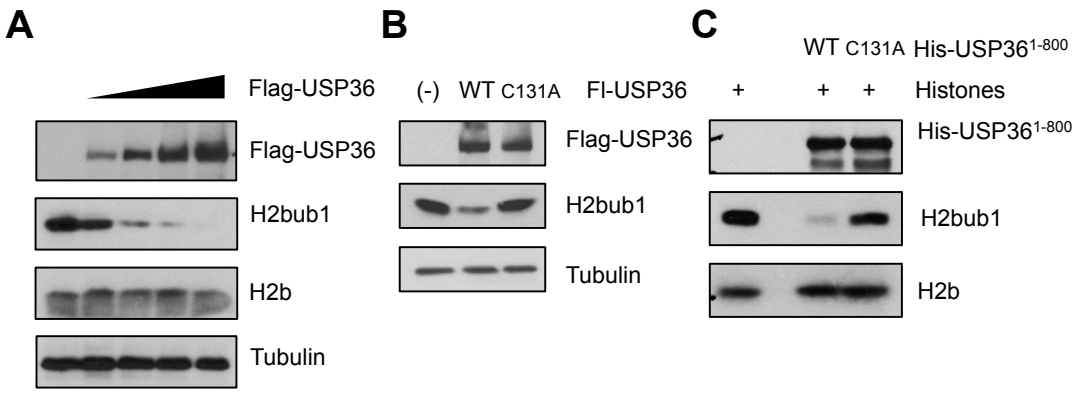

Figure 2-1: USP36 Deubiquitinates monoubiquitinated histone 2b.

(A) USP36 decreases H2bub1 in dose dependent manner. H1299 cells were transfected with increasing doses of Flag-USP36 and assayed for H2bub1 and total H2b levels. (B) USP36 mediated decreases in H2bub1 are dependent on its catalytic activity. H1299 cells were transfected with wildtype (WT) Flag-USP36 or the Flag-USP36^{C131A} (C) USP36 deubiquitinates H2bub1 in vitro. Histones were purified from H1299 cells and incubated overnight with either His-USP36$^{1-800}$ or His-USP36$^{1-800}$ purified from bacteria and assayed for H2bub1 and H2b.

interacting with histone 2b. In an in-vitro GST pull-down assay, histones purified from H1299 cells were incubated with bacterially purified GST-USP36 or GST only as a control, followed by IB analysis (Figure 2-1A). As shown in Figure 2-2A H2b bound GST-USP36, but not GST alone. Interestingly, we only detected the pulldown of H2bub1 by the GST-USP36^{C131A} mutant, but not the wild-type GST-USP36 (Figure 2-2B). This result further supports the USP36 activity to deubiquitinate H2b (Figure 2-1), as in this setting, wild type USP36 is capable of

removing the modification. To test whether endogenous USP36 associated with chromatin, nuclei isolated from H1299 cells were treated without or with micrococcal nuclease (Mnase) Mnase cleaves DNA surrounding nucleosomes, resulting in solubilization of chromatin bound factors (Figure 2-2C). In the absence of MNase, the majority of endogenous USP36 was found in the insoluble nuclear pellet, whereas in nuclei treated with MNase, the majority of USP36 was solubilized, suggesting that a significant fraction of USP36 is associated with chromatin (Figure 2-1C). Finally, to test if USP36 interacts with H2b in cells, we performed co-immunoprecipitation (co-IP) assays. Lysates from H1299 cells transfected with V5-USP36 alone or together with Flag-H2b were immunoprecipitated with anti-Flag antibody followed by IB. As shown in Figure 2-2D, V5-USP36 was immunoprecipitated with Flag-H2b only when both proteins were co-expressed. Conversely, endogenous H2b also co-immunoprecipitated with Flag-USP36$^{1-800}$, a catalytically-active that localizes to the nucleoplasm (Figure 2-2E) (Endo, Kitamura, et al. 2009). Thus, we conclude that USP36 interacts with H2b in cells and in vitro and that this is likely within the context of chromatin. This USP36-H2b interaction is likely direct, but we can not rule out the possibility of an indirect interaction mediated by unknown chromatin associated factors, as the histones were isolated from cultured cells (Figure 2-1A).

USP36 regulates the expression of p21. In order to test the function of USP36 deubiquitination of H2bub1 in regulating gene expression, we focused on the CDKN1A (gene coding for the cell cycle inhibitor p21) locus as it has be widely

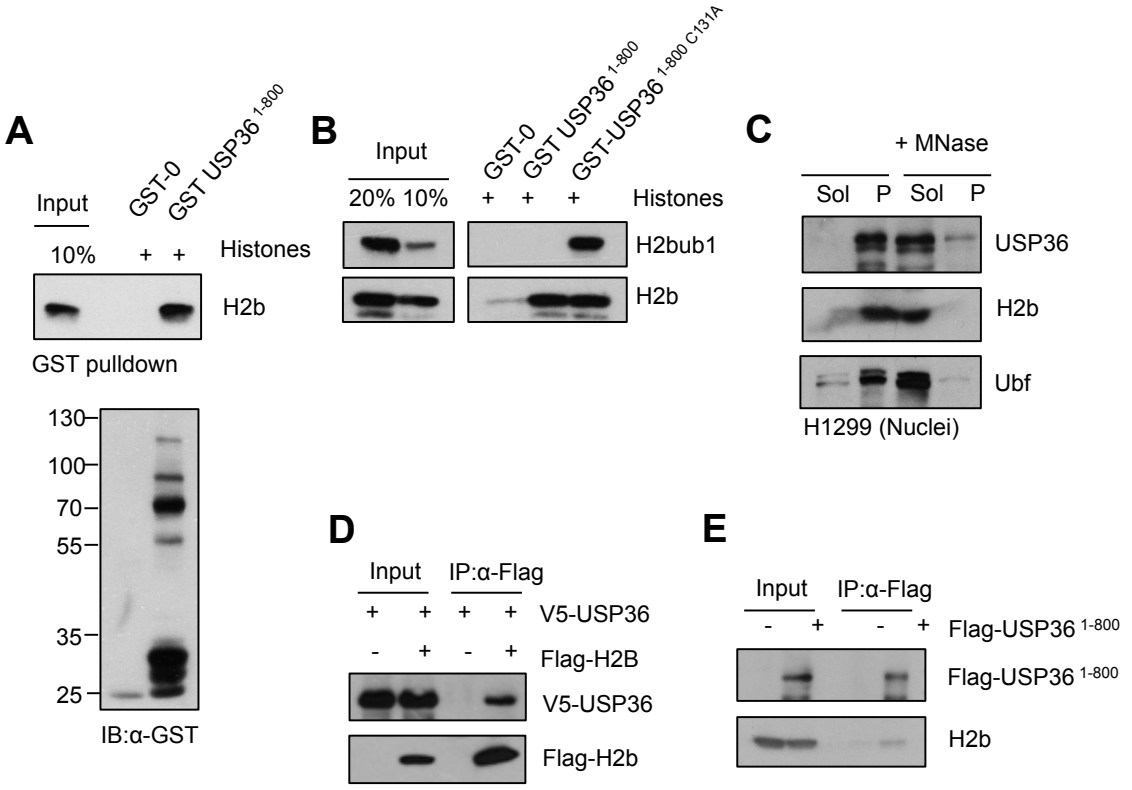

Figure 2-2: USP36 interacts with Histone 2b.

(A) Histones purified from H1299 cells were incubated with either the empty vector GST-0 or GST-USP36 $^{1-800}$, followed by GST pulldown and assayed for H2b. (B) Histones purified from H1299 cells were incubated with GST-USP36 $^{1-800}$ or GST-USP36 $^{1-800\ C131A}$ and assayed for both H2b and H2bub1. (C) USP36 fractionates with chromatin. Histones purified from H1299 cells were incubated overnight with either His-USP36 $^{1-800}$ or His-USP36 $^{1-800\ C131A}$, followed by GST pulldown. The soluble (sol) and insoluble (P) fractions of H1299 nuclei were separated in the presence or absence of micrococcal nuclease (MNase). (D) H2B and USP36 interact in cells. V5-USP36 and Flag-USP36 were co-transfected into H1299 cells followed by Flag immunoprecipitation (E) Overexpressed Flag-USP36 interacts with H2b. Hela cells were transfected with Flag-USP36, followed by Flag IP.

used as a model to study the function of H2bub1 (Kim et al. 2009; Gomes et al. 2006; Zhang & Yu 2011). Previous studies have indicated that upon p21 induction, there is a concomitant increase in H2bub1 levels within the gene body. Temporally, this increase tracks with RNA polymerase II binding (Minsky et al. 2008). Furthermore, *p21* is often epigenetically silenced in cancer through various mechanisms (Geyer 2010; Ghantous et al. 2012; Mateen et al. 2012). To determine if *p21* was an adequate model to test our hypothesis, we first examined whether there were changes in p21 levels following knockdown of USP36. RT-PCR analysis indeed showed that knockdown of USP36 by two individual lentiviral encoded shRNAs increased the levels of p21 mRNA by two-folds (Figure 2-3A) in H1299 cells. Consistently, the levels of p21 protein were also significantly increased upon USP36 knockdown (Figure 2-3B). These effects were not cell-type specific, as similar results were also observed in other tested cell lines such as Hela and U2OS cells (Figure 2-3C). These results are consistent with the function of USP36 in deubiquitinating H2b.

USP36 locally binds to the gene body of the p21 locus. As previously discussed, H2bub1 is associated with active transcription and often enriched within the gene bodies of actively transcribed genes (Kao et al. 2004; Minsky et al. 2008; Zhu et al. 2005). Given that knockdown of USP36 resulted in increased p21 levels (Figure 2-3A), we hypothesized that p21 was a target negatively regulated by USP36, potentially through deubiquitination of H2bub1. In order to examine whether USP36 specifically interacted with the p21 gene, we carried out chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR)

assays using primer sets amplifying the upstream, middle or end region of p21 (Figure 2-4A). Consistent with published results, we also observed that H2bub1 is enriched in the p21 gene body (Figure 2-4B) (Minsky et al. 2008). Next, we transfected 293 cells with either Flag-tagged wild-type USP36 or Flag-USP36 ^{C131A}, followed by IP with anti-Flag antibody and interrogation of USP36 binding to the p21 gene locus. As shown in Figure 2-4C, wild-type USP36 bound to the middle region of p21, while the catalytic mutant was bound to all three regions tested, although it was still mostly enriched in the middle region of p21. This result indicates that USP36 is enriched in the region with highest H2bub1 levels. The stronger binding affinity of the USP36 ^{C131A} mutant to the p21 gene body as well as the spectrum spanning the whole p21 gene compared to wild-type USP36 might be due to enzymatic trapping mechanism that retain the mutant on chromatin (Nicassio et al. 2007).

USP36 deubiquitinates H2bub1 within the p21 gene body. Knowing that USP36 binds to the p21 gene body, we next sought to determine whether it regulated H2bub1 levels at this locus. H1299 cells were infected with lentiviruses encoding shRNA against USP36, followed by ChIP using anti-H2bub1 antibody. As shown in Figure 2-5A, knocking down USP36 substantially increased H2bub1 levels within the middle region of p21 with only modest effects on region just downstream of the transcriptional start site and the far 3' end of CDKN1A. Consistently, overexpression of USP36 modestly, but significantly ($P<0.05$), reduced the levels of H2bub1 in the middle region of the p21 gene (Figure. 2-5B).

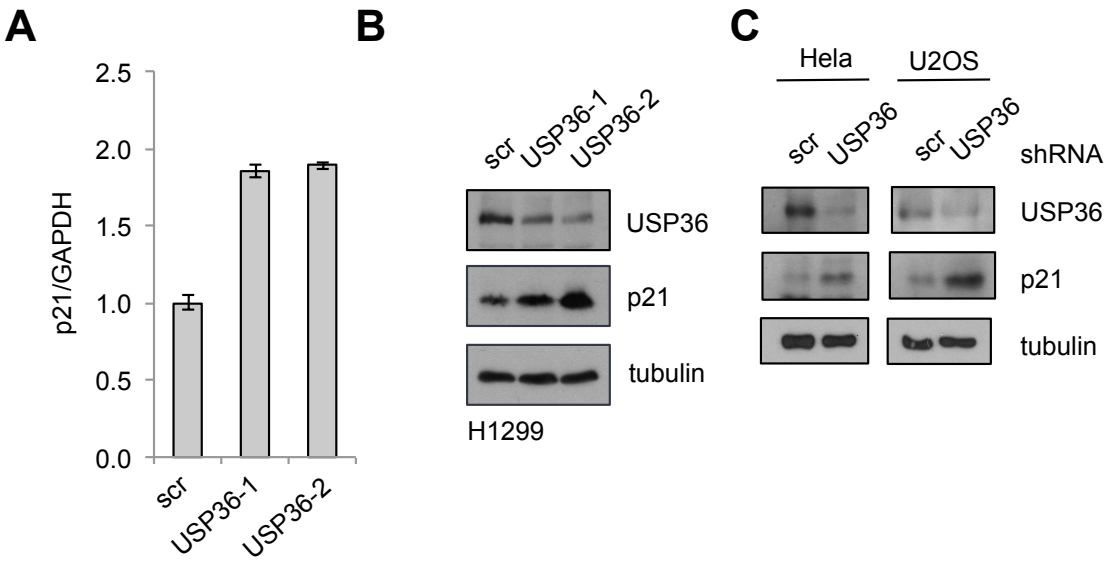

Figure 2-3: USP36 regulates expression of p21.

(A) Depletion of USP36 increases p21 mRNA. USP36 was knocked down in H1299 cells, followed by qPCR for p21 mRNA. (B) Depletion of USP36 increases p21 protein in H1299 cells. H1299 cells infected with lentivirus containing shRNA constructs targeting USP36 were harvested for immunoblot. (C) Depletion of USP36 increases p21 protein. Western blot for p21 in Hela and U2OS cells following knockdown of USP36.

These data suggest that USP36 negatively regulates p21 expression through deubiquitination of H2b at the gene body. Yet, USP36 knockdown did not alter global levels of H2bub1 in H1299 cells, suggesting that the global levels of H2bub1 are most likely regulated by multiple DUBs as previously suggested (Appendix 3**)**. As it has been shown that H2bub1 is associated with active histone mark H3K4me3, particularly enriched at the 5' end of genes, we next assayed the H3K4me3 modification in p21 gene locus. Our results showed an increase in

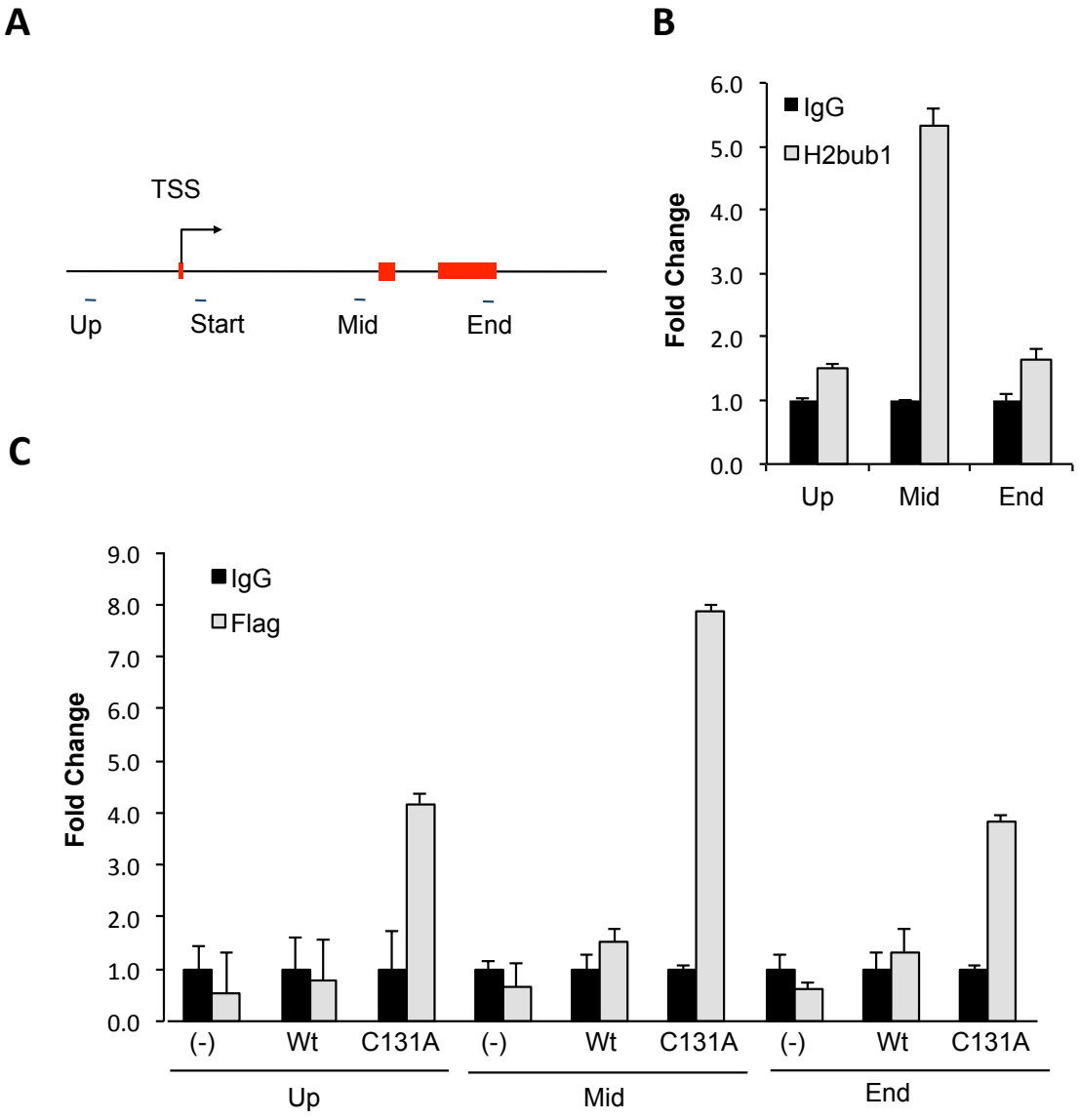

Figure 2-4: USP36 interacts with the middle region of the p21 gene.

(A) A schematic of the CDKN1A locus with the location of primers used in ChIP. (B) H2bub1 levels are highest within the gene body. H2bub1 ChIP followed by qPCR with listed primers. (C) Overexpressed USP36 interacts with the p21 gene. 293 cells were transfected with either Flag-USP36 or Flag-USP36 ^{C131A}, followed by Flag ChIP and detection of various sites throughout the p21 gene by qPCR.

H3K4me3 at the region downstream from the start site **(**Figure 2-6A) (Kim et al. 2009; Kim et al. 2013). We also detected slightly increased global levels of H3K4me3 (Figure 2-6B**)**. In contrast, H3K36 methylation, which is not thought to be involved in histone cross-talk, remained unchanged **(**Figure 2-6A**)**.

Knockdown of USP36 decreases proliferation. Previous studies have shown that H2bub1 levels are negatively correlated with cancer progression, suggesting that there is deregulation of the enzymes regulating this modification (Z. J. Wang et al. 2013; Prenzel et al. 2011). Consistently, it was discovered that RNF20 was silenced through hypermethylation in breast cancer (Prenzel et al. 2011). Furthermore, RNF20 has tumor suppressive properties as shown by increased xeongraft burden and migratory capacity upon knockdown of RNF20 (Prenzel et al. 2011). In contrast, USP22, an H2bub1 deubiquitinase, supports oncogenesis and remarkably was shown to be a negative prognostic factor in a “death by cancer gene signature” (Glinsky 2005). To analyze whether USP36 is also required for cell growth, USP36 was knocked down using two different shRNAs for 48 hours followed by cell proliferation assay using the Incucyte Zoom system to monitor for cell confluence over time. Both shRNAs reduced the confluence of H1299 cells by more than 50% at the end of the assay (Figure 2-7A). To further support this observation we conducted an MTS assay in H1299 cells and compared viability of a scrambled to USP36 shRNA transfected cells (Figure 2-7B). As shown in Figure 2-7B, knockdown of USP36 significantly decreases the viability. To determine whether some of this effect was due to decreased

proliferation, we performed cell proliferation assays using EDU labeling. Cells that have entered or passed through S phase will incorporate EDU in their DNA.

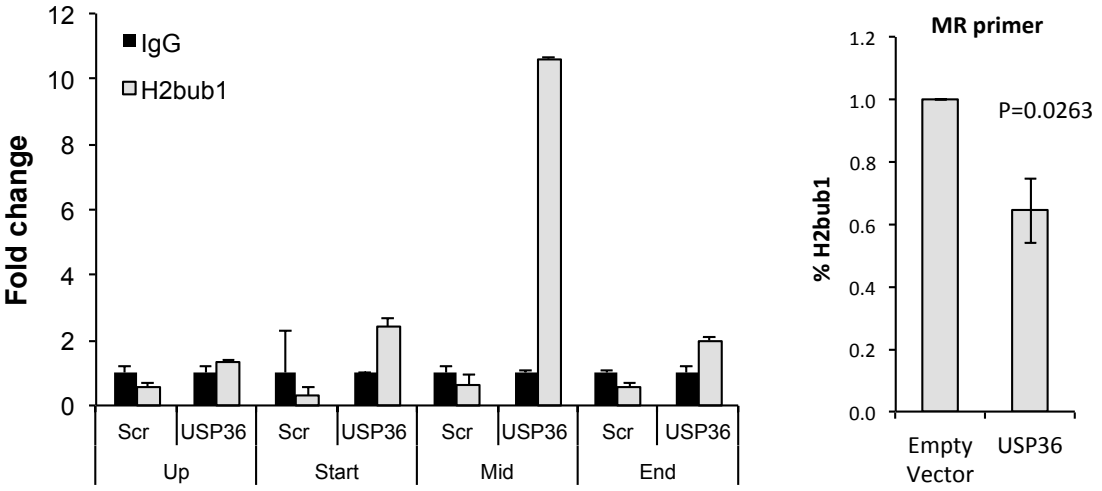

Figure 2-5: USP36 Deubiquitinates H2b-Ub within p21 gene.

(A) Depletion of USP36 results in increased H2bub1 specifically in the gene body. 293 were transfected with wt-USP36 and the levels of H2bub1 were assayed by ChIP using the p21 (mid primer pair). (B) Overexpression of USP36 decreases H2bub1 in the middle region of p21. H1299 cells were infected with lentivirus containing sh-RNA against USP36. This was followed by ChIP for H2bub1 throughout the p21 gene.

In order to test this, H1299 cells were infected with scrambled or USP36 shRNA lentiviruses for 72 hours, followed by pulse-labeling with EDU for 4 hours (Figure 2-7C). The total number of EDU positive cells was reduced by ~40% following USP36 knockdown, suggesting a proliferaton defect that is consistent with the increased p21 expression.

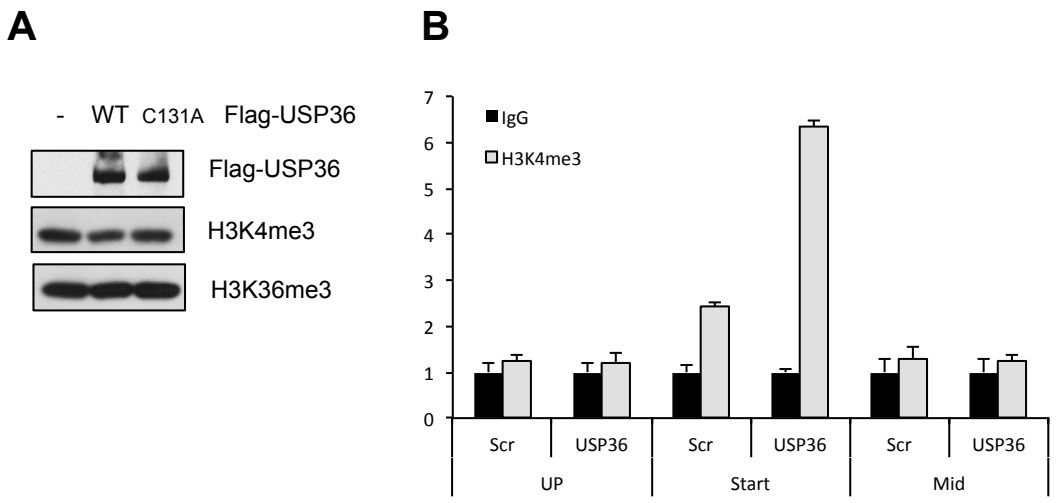

Figure 2-6: USP36 deubiquitination of H2bub1 modestly effects H3K4me3.

(A) Overexpression of USP36 causes minor decrease in H3K4me3. Flag-USP36 wild type or FLag-USP36^{C131A} was transfected into H1299 cells, followed by assay for H3K4me3 and H3K36me3 by immunoblot. (B) Depletion of USP63 results in a slight increase in H3K4me3 at the transcriptional start site. H1299 cells were infected with lentivirus containing sh-RNA against USP36 or a control. ChIP analysis was done with anti-H3K4me3 and various primers at the p21 locus were assayed.

Knockdown of USP36 induces cell senescence. Next we tested whether the induced p21 upon USP36 knockdown was also functional to regulate senescence (Campisi & d'Adda di Fagagna 2007; Romanov et al. 2010). Here, primary lung fibroblast WI38 cells were infected with scrambled or USP36 shRNA lentiviruses, followed by beta-galactosidase staining (Debacq-Chainiaux et al. 2009) . As shown in Figure 2-8A and consistent with previous

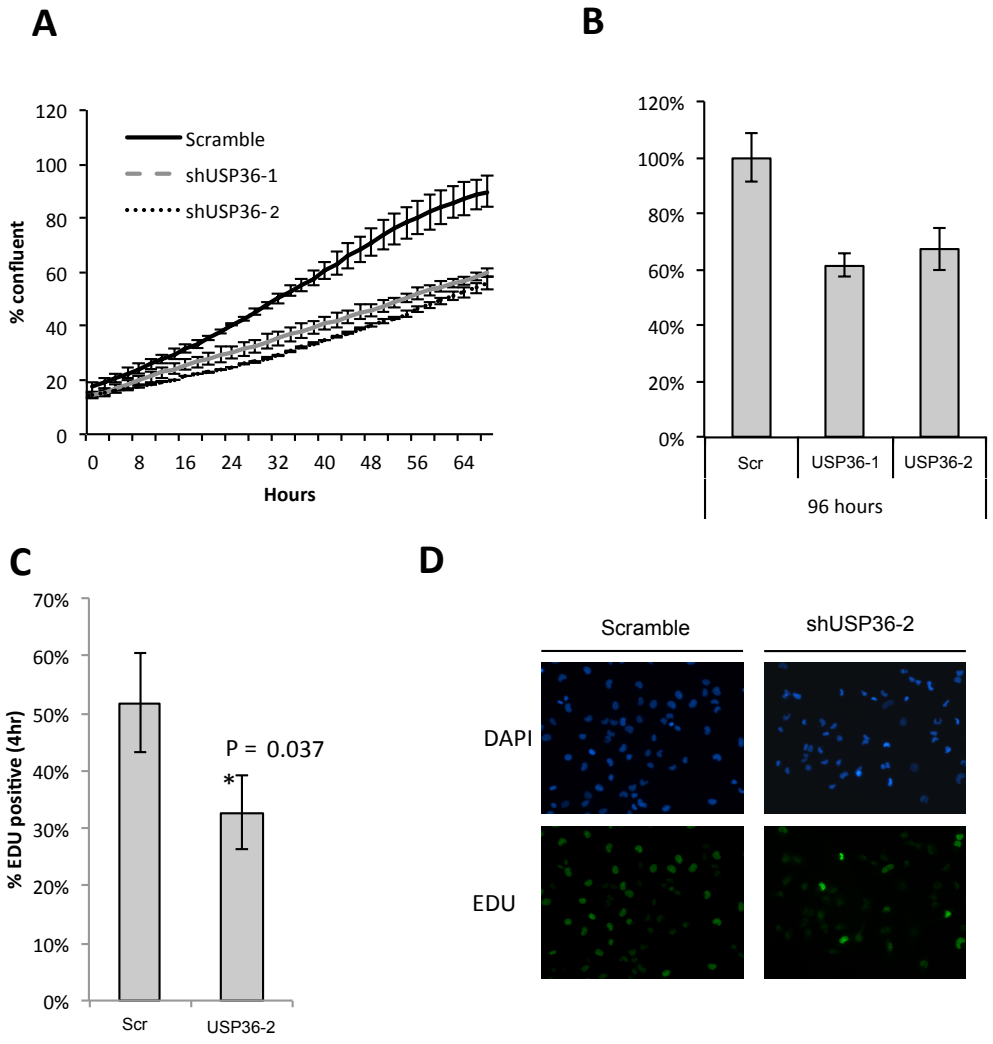

Figure 2-7: Depletion of USP36 decreases proliferation.

(A) H1299 were infected with lentivirus containing scramble shRNA or shRNAs targeting USP36. At 48 hours they were replated and assayed for confluence using the incuCyte zoom, which collected images every 2 hours. (B) Depletion of USP36 reduces cell viability. USP36 was knocked down in H1299 cells followed by MTS assay. (C) USP36 decreases proliferation. USP36 was knocked down in H1299 cells followed by a 4 hour pulse with EDU. The % EDU positive nuclei was calculated. (D) Representative image of (C)

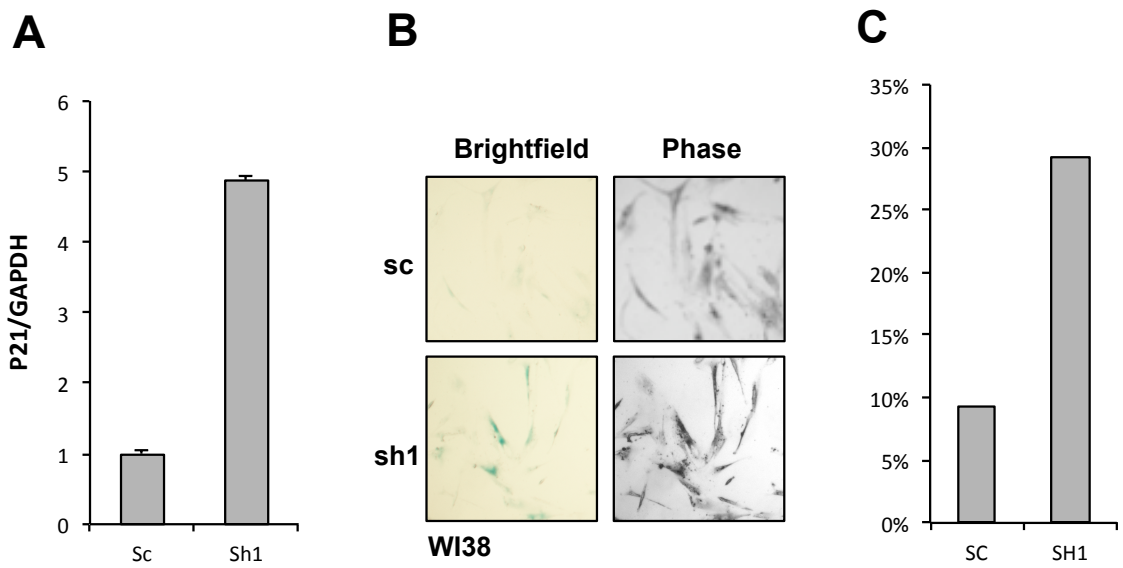

Figure 2-8: Loss of USP36 induces senescence.

(A) qPCR for p21 mRNA following knockdown of USP36 in WI38 cells. (B) WI38 cells were infected with lentivirus expressing either a scrambled control or sh-RNA targeting USP36 for 7 days. Cells were then fixed and stained for the senescence marker beta-galactosidase. Shown are representative brightfield images alongside their corresponding and phase images. (C) Quantification of beta-glaactosidase staining in either the scramble control or sh-RNA knockdown of USP36.

results, knockdown of USP36 also significantly increased the levels of p21 mRNA in WI38 cells. This coincided with a 3-fold increase in cells with beta-galactosidase staining, a marker of senescence (Figure 2-8B,C). Altogether, our data suggest that USP36 may play a role in oncogenesis in part by suppressing senescence through repression of p21.

2.4 DISCUSSION

DUBs regulate virtually all aspects of ubiquitin biology with effects ranging from altered protein stability to changes in protein function and modified gene expression (Devine & Dai 2013; Nijman et al. 2005). Increasingly, DUBs are being appreciated as key regulators of various pathologies such as cancer (Fraile et al. 2012). Thus targeting DUBs in a therapeutic context may be advantageous due to their druggable catalytic domains and broad effects on number of substrates, which often regulate multiple nodes within the same pathway.

One key DUB substrate is H2bub1 that plays an important role in modulating chromatin dynamics and gene expression (Fierz et al. 2011; Fuchs et al. 2012). While many DUBs are known to regulate H2bub1 in higher eukaryotes, a human ortholog to yeast ubp10 has not been identified. Here we show that USP36, like its drosophila and yeast orthologs Scrawny and Ubp10, can act to regulate gene expression through deubiquitinating H2bub1 (Gardner et al. 2005; Buszczak et al. 2009). We show that USP36 interacts with H2b and deubiquitinates H2bub1 both in cells and in vitro. This interaction likely occurs within the context of chromatin as we found that a significant pool of USP36 is enriched on chromatin. Whether this USP36-H2b binding is direct or through other partner proteins on chromatin still needs to be clarified. If the binding is indirect, one potential candidate through which USP36 accesses H2bub1 is RNF20, the ubiquitin ligase for monoubiquitinating H2B, as we found that USP36 interacts with RNF20 in cells determined by co-IP assays (Appendix 5). While we

clearly show the drastic reduction of global H2bub1 by USP36 overexpression, we did not observe a significant global increase upon knockdown of USP36 (Appendix 3). This phenomenon is most likely because there are numerous DUBs that regulate global levels of H2bub1 in humans. Similarly knockdown of USP22 does not result in dramatic increases in H2bub1 (Johnsen 2012). Nevertheless, using the p21 gene locus as a model, we clearly show by ChIP-qPCR analysis that USP36 is bound to the gene body of the p21 locus, correlating with a reduction of H2bub1 upon USP36 knockdown. At expression level, we show that knockdown of USP36 markedly increased the levels of p21 mRNA and protein. This correlates with increased H2bub1 levels within the coding region of p21 and suggests a repressive role of USP36 in the p21 locus (Figure 2-9). To our knowledge, this is the first example of a human DUB that negatively regulates target gene expression by antagonizing H2bub1 deposition within the gene body.

Functionally, we show that knockdown of USP36 significantly inhibited cell proliferation in cancer cells and induced cell senescence in primary fibroblast cells and these effects are correlated with the drastic induction of p21 expression. In addition, USP36 is a nucleolar DUB and has recently shown to play a critical role in ribosomal biogenesis through various mechanisms. First, it was shown to regulate the stability of Nucleophosmin (NPM) and Fibrillarin (FBL), two critical nucleolar proteins that are implicated in ribosomal RNA (rRNA) processing (Endo, Kitamura, et al. 2009; Endo, Matsumoto, et al. 2009). It also regulates the stability of the largest RNA polymerase I subunit RPA194 which is consistent with a reduction of pre-rRNA synthesis upon depletion of USP36

(Peltonen et al. 2014; Endo, Kitamura, et al. 2009). Third, we recently found that USP36 deubiquitinates and stabilizes c-Myc, a key oncogene required for cell proliferation and a master regulator of ribosomal biogenesis (Sun et al. 2015). Together with its role in silencing the p21 gene, USP36 is clearly a key regulator of cell growth and proliferation. Consistent with this notion, we have shown USP36 is essential to the growth of various cancer cell lines. A wide spectrum of cancers harbor amplifications and/or show overexpression of USP36. Additionally, we and others have observed that USP36 is overexpressed in several tested primary human cancers (Li et al. 2008; Sun et al. 2015; Cerami et al. 2012).

Given that USP36 primarily localizes to nucleoli, it will be interesting to test whether it regulates rRNA synthesis by deubiquitinating H2b at the rDNA loci at the nucleolus. We do find that H2bub1 levels are significantly lower in the nucleolus compared to that in the nucleoplasm, which may be regulated by the presence of USP36. Although mostly nucleolar, we hypothesize that its nuclear regulation of RNA polymerase II is likely through an actively population that traffics through the nucleolus and the nucleoplasm (Figure 2-9A,B). Together, these observations suggest that USP36 may have oncogenic activity within the nucleus, at least through its ability to repress p21.

Like USP36, USP22 is predicted to have oncogenic function and is part of a poor prognosis signature. Thus, determining the relationship between USP36 and other DUBs regulating H2bub1, particularly USP22, will be interesting to flesh out. The target repertoire of USP36 is likely smaller than that of USP22, but

may expand under conditions that regulate localization changes, such as deregulated NPM (Endo, Kitamura, et al. 2009). Global analysis of USP36's impact on H2bub1 will be required to answer this question. It is possible that different DUBs either regulate distinct target genes or regulate H2bub1 based on its position within a gene, as has been shown in yeast where ubiquitination at promoters inhibits recruitment of the basal transcription machinery but promotes elongation within the gene body (Schulze et al. 2011). Another possibility is that regulation of multiple DUBs at overlapping targets could be a mechanism for fine-tuning expression at a particular locus. For example, USP22 was shown to promote transcription of p53 target genes, in particular p21 (Zhang et al. 2008). In this context, the role of USP22 and USP36 are seemingly opposed. However, it has been established that cycling of H2bub1 is important for maximal transcription at loci that it regulates. Furthermore, a recent study has suggested that SAGA may be recruited through H2bub1 in order to promote transcription. USP36 mediated deubiquitination of H2bub1 at p21 may interfere with SAGA recruitment and thus the dynamics of H2bub1. Future studies should not only clarify this point but should also aim to integrate the various DUBs regulating H2bub1 into a larger framework.

In summary, we have found that USP36 is a novel H2b DUB, further supporting it as the human ortholog of the yeast ubp10 and Drosophila Scrawny. In addition, similar to Scrawny (dUSP36) in drosophila, human USP36 acts as an inhibitor of autophagy and a slow growth phenotype in the ubp10 deletion yeast

strain can be rescued by expressing human USP36 (Buszczak et al. 2009; Taillebourg et al. 2012). Given that USP36's overexpression has such a dramatic

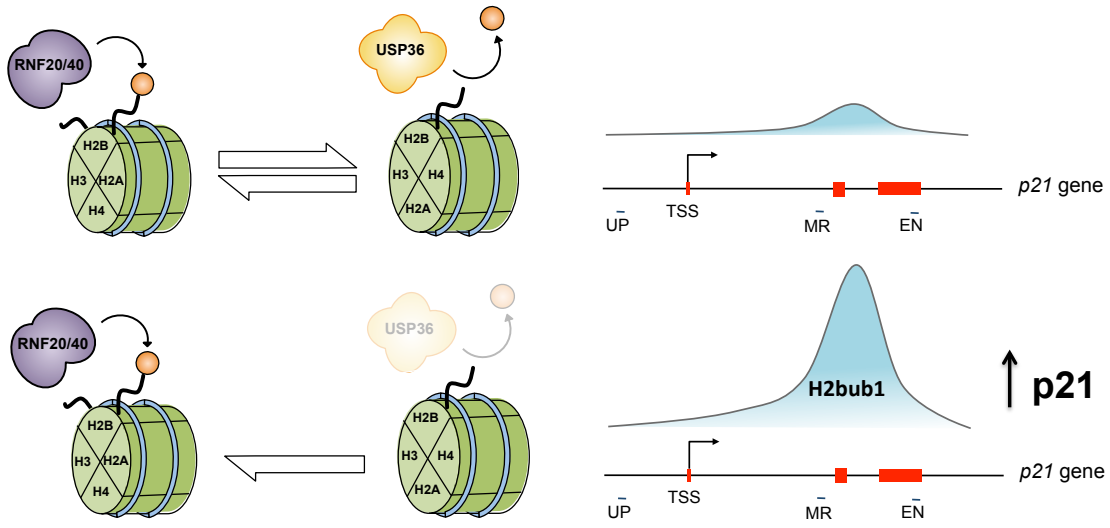

Figure 2-9: Model USP36 regulates H2bub1 at the p21 locus.

In arrested cells (not shown) that express p21, RNF20 mediated monoubiquitination of H2b is favored. However, in proliferative cancer cells, in which USP36 is overexpressed, the balance shifts at the p21 locus in favor of deubiquitination (Top panel). Decreasing USP36 levels in proliferative cells shifts the balance back in favor of RNF20 mediated mono-ubiquitination of H2b and re-expression of the cell cycle inhibitor p21.

impact on global H2bub1 levels, it is not unreasonable to think that it may be acting in a similar manner when overexpressed or amplified. A negative correlation between global levels of H2bub1 and cancer progression has been

observed, our study uncovers yet another oncogenic function for USP36 and justifies its consideration as therapeutic target observed in breast, colon, and gastric cancer (Prenzel et al. 2011; Wang et al. 2015; Z. J. Wang et al. 2013). This is consistent with USP36's roles as an oncogene in the cell.

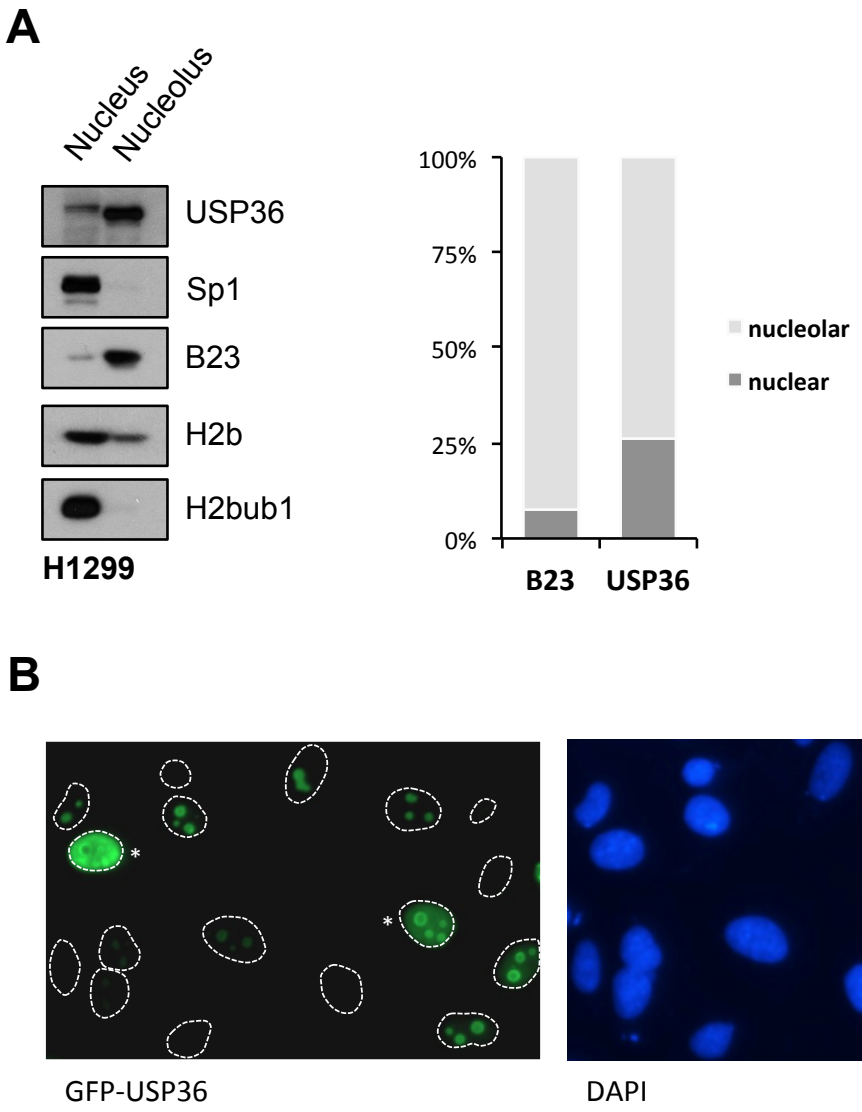

Figure 2-10: A small pool of USP36 localizes to the nucleoplasm.

(A) The nuclei of Hela cells were further fractionated into the nucleoplasmic and the nucleolar fractions and assayed for USP36, SP1 (a nuclear marker), B23/NPM (a nucleolar marker), H2b and H2bub1 by IB. The amount of nucleoplasmic vs nucleolar USP36 and B23/NPM is quantified (right panel). (B) GFP-USP36 was transfected into H1299 cells and visualized under an IF microscopy (shown on left). Dapi staining of the nucleus is shown on the right.

3 USP36 is a novel nucleolar deubiquitinating enzyme for the p53-MDM2 Loop

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3.1 ABSTRACT

Deubiquitination is an important mechanism regulating the p53-MDM2 axis (Devine & Dai 2013). Increasingly more deubiquitinating enzymes (DUBs) are being identified that control the stability of p53 either by acting on it directly or by affecting its regulators. These DUBs include nuclear localized USP7 and USP2 as well as cytoplasmic USP10 and Otub1 (X.-X. Sun et al. 2011; Li et al. 2002; Stevenson et al. 2007; Yuan et al. 2010). The nucleolus is a critical hub for linking different types of cellular stress to the regulation of ribosome biogenesis and conversely signaling back to other cellular compartments when under stress itself (Rubbi & Milner 2003). Furthermore, it plays a critical role in regulating the stability of p53 through the ubiquitin proteasome system (UPS). Understanding the dynamics of this regulation within the nucleolus, including the conditions under which DUBs regulate the p53 pathway is critical. In spite of this, a nucleolar DUB regulating the p53-MDM2 signaling axis has not been identified. Here, we show that the nucleolar USP36 is a novel regulator of the p53-MDM2 pathway. USP36 interacts with both MDM2 and p53 in cells and *in vitro*. It deubiquitinates and stabilizes both proteins, leading to the increased levels of both proteins upon USP36 overexpression. These results reveal USP36 as a novel nucleolar DUB that regulates both MDM2 and p53. Future studies will determine the physiological significance of this regulation.

3.2 INTRODUCTION

p53 is a sequence-specific transcription factor that regulates expression of many target genes. Broadly, the biological function of p53 is to induce cell cycle arrest, apoptosis, or senescence in response to diverse stress, thereby preventing cells from undergoing malignant transformation (Kruse & Gu 2009; Levine & Oren 2009; Vogelstein et al. 2000; Vousden & Prives 2009). Twenty years ago, p53 was famously coined "guardian of the genome", referencing its ability to stall cell cycle progression in the face of DNA damage and to initiate either repair or death (Lane 1992). Since that time, it has become clear that loss of p53 function is practically a universal feature of cancer. Germ-line mutations in p53 are found in the familial Li-Fraumeni syndrome, which is characterized by early-onset cancers in diverse tissues (Malkin et al. 1990; Srivastava et al. 1993). Thus, p53 plays an essential role in protecting the organism from cancer.

p53 possesses an N-terminal, bipartite acidic transactivation domain, which makes contacts with basal transcription factors and co-activators allowing for the initiation of transcriptional activation at target genes (Beckerman & Prives 2010; Fields & Jang 1990; Raycroft et al. 1990). The central DNA-binding domain mediates sequence-specific binding to target gene promoters (Bargonetti et al. 1993; Pavletich et al. 1993; Wang et al. 1993). Of the hundreds of *p53* missense mutations identified in human cancers, the vast majority are found within this central DNA-binding domain, suggesting that its role as a transcription factor is essential for its tumor suppressive functions (Soussi et al. 2000; Wang et al. 1993). While the spectrum of mutations have varying degrees of phenotypic

effects, they generally fall into two classes: those that disrupt residues required for making contacts with DNA and those that alter protein conformation and folding, which preclude DNA binding or result in decreased stability. p53 also contains several unique domains in its C-terminus. This includes a tetramerization domain that facilitates tetramer formation, allowing for optimal transcriptional activity (Wang et al. 1993). There is also a basic regulatory region in the far C-terminus that is required for transcriptional activation of specific target genes (Bayle et al. 1995; Jayaraman & Prives 1995; McKinney et al. 2004). This region is also involved in recruiting co-factors, such p300, that acetylates histones and promotes an open chromatin state (Espinosa & Emerson 2001).

Upon activation, p53 binds to its cognate DNA response elements (REs) and directly regulates gene transcription. More than 4000 putative p53 REs are present within the genome (Wang et al. 2001). While the vast majority of these sites have yet to be experimentally validated, attempts are being made to narrow the list by increasing the stringency of criteria required to define bona fide p53 targets. Using a more rigorous definition, ~129 high confidence targets have been identified (Riley et al. 2008). Although the picture is increasing in complexity, many canonical targets have been well characterized for each of the p53 output programs. For example, the p53 target gene, CDKN1A that codes for p21, is a cyclin dependent kinase (CDK) inhibitor that induces G1 arrest. A second program for eliminating permanently damaged cells is through the induction of apoptosis. Many pro-apoptotic p53 targets have been identified including those involved in the intrinsic apoptotic pathway, such as BAX, NOXA,

PUMA, p53AIP1 and BID (Miyashita et al. 1994; K. Oda et al. 2000; Nakano & Vousden 2001; Sax et al. 2002; E. Oda et al. 2000).

The detrimental effects of p53 activation make it essential to hold the protein at low levels under non-stressed conditions in normal cells. This is mainly achieved through its interaction with the ubiquitin ligase MDM2, which is assisted by its partner protein MDMX. MDM2 mediates ubiquitination of p53 and targets it for proteasomal degradation. In response to stress, MDM2 ceases to ubiquitinate p53. This results in its stabilization and subsequent activation. In addition to a primary role in targeting p53 stability, MDM2 also binds to the transactivation domain of p53 through its N-terminus, which confers the ability to directly inhibit its activity (Honda et al. 1997; Momand et al. 1992). Furthermore MDM2 can promote p53 nuclear export and suppress the co-activator p300 from acetylating it (Freedman & Levine 1998; Geyer et al. 2000; Roth et al. 1998; Ito et al. 2001; Kobet et al. 2000). It is therefore unsurprising that MDM2 is overexpressed or amplified in many human cancers that retain wild-type p53 (Bueso-Ramos et al. 1993; Cordon-Cardo et al. 1994; Dworakowska et al. 2004; Momand et al. 1998; Oliner et al. 1992).

MDM2 is a p53 target gene that is upregulated in order to attenuate the toxic consequences of p53 once the cellular stress is resolved. This allows p53 to feedback on itself (Barak et al. 1993; Wu et al. 1993). *In vivo* studies have verified this feedback loop by showing that a genetic knockout of p53 rescues the lethal phenotype of *mdm2* knockout mice (Jones et al. 1995; Montes de Oca Luna et al. 1995). Furthermore, mice homozygous for a knock-in of an MDM2

E3-inactive mutant, C462A, are also embryonic lethal and can be rescued by deleting p53 as well (Itahana et al. 2007). Interestingly, this mouse model also challenges the classical dogma that MDM2 is its own E3 ligase, as the RING appears entirely dispensable for MDM2 degradation. However, the E3 function of MDM2 is indispensible for its suppression of p53 *in vivo* (Itahana et al. 2007).

MDMX is a homolog of MDM2, as well as its binding partner (Shvarts et al. 1996). Like MDM2, MDMX also interacts with the transactivation domain of p53 through its highly conserved N-terminus and interferes with p53 transcriptional activity (Gu et al. 2002; Marine & Jochemsen 2005; Linares et al. 2003). MDM2 forms either homodimers or heterodimers with its partner MDMX through the conserved function of their C- terminal RING domains (Tanimura et al. 1999; Sharp et al. 1999; Gu et al. 2002). Although MDMX contains a RING domain that is structurally similar to that of MDM2, it does not possess significant E3 activity (Stad et al. 2001; Jackson & Berberich 2000). In spite of this, we know that MDMX is required for proper regulation of p53, both in the presence and absence of stress. While it is not entirely clear how MDMX functions, several lines of evidence suggest that it modulates MDM2's activity. The MDM2 homodimer is relatively unstable due to its autoubiquitinating activity. When partnered with MDMX, on the other hand, the heterodimer is stable and selectively shifts ubiquitination from MDM2 towards p53 (Linares et al. 2003; Linke et al. 2008). Studies have also shown that MDMX may enhance the E3 activity of MDM2 through residues in its extreme C-terminus. These residues, which are located outside of the RING domain of both MDM2 and MDMX, play a critical role in

mediating E3 activity (Linke et al. 2008; Poyurovsky et al. 2007; Uldrijan et al. 2007). An MDM2 point mutant (Y489A) that shows no E3 ligase activity can be rescued by forming heterodimers with MDMX. Interestingly the rescue is dependent on residues located within the extreme C-terminus of MDMX outside of its RING domain. This suggests that this region of MDMX can somehow cooperate with the RING domain of MDM2 to effect E3 function (Uldrijan et al. 2007). MDMX has also been suggested to influence the site of ubiquitin conjugation, with a preference for C-terminal lysines in p53, whereas in its absence ubiquitin is directed to lysines within the DNA binding domain, at least *in vitro* (David et al. 2011). This may be a result of MDMX's ability to induce conformational changes in p53 bringing different portions of the protein in contact with the E2 (David et al. 2011). The C-terminus of MDMX may also recruit specific E2s that will determine the type of ubiquitin linkage and thus the fate of the substrate (Wade et al. 2010). Supporting the essential role for MDM2-MDMX heterodimerization in regulating p53 *in vivo*, mice homozygous for a knock-point mutation in MDMX (C462A) are embryonic lethal. This is rescued by concomitant deletion of the *p53* gene (Huang et al. 2011).

Numerous stress signals, including, but not limited to, DNA damage, hypoxia, telomere erosion, aberrant oncogene activation and ribosomal stress are known to induce p53, predominantly by inhibiting negative regulation imposed by MDM2/MDMX (Vousden & Lane 2007). DNA damaging agents induce and activate p53 primarily through the induction of a number kinases, notably Ataxia telangiectasia mutated kinase (ATM), ataxia telangiectasia RAD3-

related kinase (ATR), Chk1 and Chk2 which phosphorylate p53, MDM2, MDMX (Banin et al. 1998; Canman et al. 1998; Hirao et al. 2000; Khosravi et al. 1999; Maya et al. 2001; Mayo et al. 1997; Shieh et al. 2000; Tibbetts et al. 1999). Early work suggested that a few critical sites may have been required for p53 activation following DNA damage. For example, phosphorylation at Ser15, Ser20 or Thr18 within the N-terminal transactivation domain can decrease the interaction between p53 and MDM2 and subsequently promote the recruitment of co-activators, such as p300 (Lambert et al. 1998).

Aberrant oncogenic activation, such as overexpression of c-Myc, Ras, and E2F, etc., induces oncogenic stress, resulting in p53 stabilization and activation (Hermeking & Eick 1994; Wu & Levine 1994; Kowalik et al. 1995; Bates et al. 1998; Serrano et al. 1997). This is mediated by the small basic nucleolar protein $p14^{ARF}$ ($p19^{ARF}$ in mouse) another important tumor suppressor frequently deleted in human cancers (Zhang & Xiong 2001). In response to aberrant proliferation and growth signals downstream of high oncogene activity, ARF binds to the central acidic domain of MDM2 and inhibits MDM2-mediated p53 ubiquitination and proteasomal degradation (Honda & Yasuda 1999; Tao & Levine 1999; Zhang et al. 1998). In addition, ARF also sequesters MDM2 into the nucleolus, although this effect is dispensable for its role in regulating p53, and suppresses nuclear export of both MDM2 and p53 (Weber et al. 1999; Tao & Levine 1999; Zhang et al. 1998; Llanos et al. 2001; Zhang & Xiong 1999).

Ribosomal stress (also called nucleolar stress) can also activate p53 (Zhang & Lu 2009). Ribosomal stress occurs following perturbation of ribosome

biogenesis and can be induced in cells with a low dose of actinomycin D, 5-florouracil, mycophenolic acid, serum starvation, or knockdown of essential nucleolar components required for ribosome biogenesis (Bhat et al. 2004; Dai et al. 2004; Fumagalli et al. 2009; Sun et al. 2007; Sun et al. 2008; Sun et al. 2010; Yuan et al. 2005). An increasing number of studies including our own have shown that several ribosomal proteins (RPs), including L5, L11, L23, L26, L6, S7, S27, S27a, S14, S25, and S26 are molecular mediators responsible for p53 activation following ribosomal stress and this number is likely increasing (Bhat et al. 2004; Dai et al. 2004; Chen et al. 2007; Dai & Lu 2004; Jin et al. 2004; Lohrum et al. 2003; Ofir-Rosenfeld et al. 2008; X. X. Sun et al. 2011; Xiong et al. 2011; Hao et al. 2009; Zhang et al. 2010; Zhang et al. 2003; Zhou et al. 2012; Zhu et al. 2009). These RPs interact with MDM2 and suppress MDM2-mediated p53 ubiquitination and degradation, leading to p53 stabilization(Zhang & Lu 2009). Clearly some RPs such as L5 and L11 are non-redundant MDM2 inhibitors, as knockdown of either L5 or L11 abolishes ribosomal stress-induced p53 activation by a broad range of treatments (Bhat et al. 2004; Dai et al. 2008; Sun et al. 2007; Sun et al. 2010; Sun et al. 2008; Morgado-Palacin et al. 2012; Fumagalli et al. 2012). By contrast, other RPs such as L23 and S14 play a redundant role in regulating p53 following ribosomal stress, as knockdown of these RPs triggers p53 activation (Dai et al. 2004; Jin et al. 2004; Zhou et al. 2012; Fumagalli et al. 2012). Why multiple ribosomal proteins regulate the MDM2-p53 loop is still not clear. Nevertheless, genetic knock-in of the L11- and L5-binding defective MDM2 mutant ($MDM2^{C305F}$) abolished ribosomal stress, but

not DNA damage, induced p53 activation and significantly accelerated Eμ-Myc-induced lymphomagenesis in mice, validating the essential role for L5 and L11 in mediating ribosomal stress-induced p53 activation *in vivo*. Interestingly, all above RPs bind to the central acidic and/or zinc finger domains of MDM2, suggesting that these centrally located regions contain critical regulatory domains that can be targeted for MDM2 inhibition(Macias et al. 2010).

Recently, there has been a surge of interest in deubiquitinating enzymes (DUBs) that regulate the p53 signaling pathway. These DUBs are a mixture of both positive and negative regulators with varying degrees of specificity and different modes of regulation. The first identified and most well-characterized p53 DUB, USP7 (also known as HAUSP for Herpes-Associated Ubiquitin Specific Protease), was identified as a p53 binding protein by an affinity purification (Li et al. 2002). Both *in vivo* and *in vitro* assays demonstrated that USP7 is a bona fide p53 DUB. Overexpression of USP7 induces p53 activation, leading to growth suppression and apoptosis. DNA damage increases the interaction between p53 and USP7 (Li et al. 2002). Shortly thereafter, MDM2 was also recognized as a target of USP7 and suggested to be the true physiological substrate. This was hypothesized based on the fact that complete knockdown or loss of USP7 resulted in dramatic p53 activation (Li et al. 2004; Meulmeester et al. 2005). Overall, in the absence of DNA damage, USP7 preferentially stabilizes both MDM2 and MDMX and MDM2 and MDMX require USP7 for stability. Upon DNA damage the interaction between MDM2/MDMX and USP7 decreases in an ATM

dependent manner (Meulmeester et al. 2005). Concomitantly, the interaction between USP7 and p53 increases.

USP10 was identified as the first p53 specific DUB because it targets p53 and not MDM2 or MDMX (Yuan et al. 2010). It normally localizes to the cytoplasm and is thought to help maintain steady state levels of cytoplasmic p53. p53 ubiquitination and nuclear export are differentially regulated by MDM2 such that low levels of MDM2 induce monoubiquitination and nuclear export of p53 whereas high levels mediate its polyubiquitination and nuclear degradation (Li et al. 2003). Under non-stressed conditions, USP10's activity toward p53 is relatively weak, probably due to its overall instability. Following DNA damage, ATM phosphorylates USP10, resulting in stabilization of USP10 and allowing it to more efficiently deubiquitinate and recycle cytoplasmic p53 back to the nucleus (Yuan et al. 2010). Also, a small portion of USP10 translocates to the nucleus to aid in p53 stabilization, presumably alongside USP7. Consistently, USP10 is significantly downregulated in renal clear cell carcinomas (Yuan et al. 2010).

In contrast, USP2a differs from USP7 and USP10 in that it deubiquitinates MDM2 and MDMX, but not p53, leading to destabilization of p53 (Allende-Vega et al. 2010; Stevenson et al. 2007). Thus, USP2a has suspected oncogenic roles and is overexpressed in a subset of prostate cancers (Priolo et al. 2006). In this context USP2a regulates invasiveness by destabilizing p53 and interferes with its ability to transactivate microRNAs that negatively regulate c-Myc (Benassi et al. 2012). A second DUB that negatively regulates p53 signaling is USP4. It differs from USP2a, in that it deubiquitinates and stabilizes a different E3 that

ubiquitinates p53 called ARF-BP1 (X. Zhang et al. 2011). Although MDM2 is the predominant E3 regulating p53, others including COP1, ARF-BP1, and Pirh2 have recently been identified (Dai et al. 2006).

The various DUBs are hypothesized to regulate p53 in response to different types of stress. For example, USP29 is transcriptionally activated following oxidative stress and then mediates p53 deubiquitination. Overexpression of USP29 stabilizes p53 and induces p53-dependent apoptosis in HCT116 cells (J. Liu et al. 2011). USP29 may be an important factor in resolving issues surrounding p53 outputs in response to oxidative stress and therefore a potentially useful biomarker (Vigneron & Vousden 2010). On the other hand, functional outcomes of p53 activation are known to vary based on the strength, duration and type of stress encountered. Slight alterations in p53 levels at any point following the induction of stress may have a dramatic impact on the response elicited. Interestingly, USP42 appears to regulate p53 levels only during an early phase of the stress response and does not affect the basal levels of p53 in unstressed cells (Hock et al. 2014). Although required for only a short window of time, USP42 is essential for a durable cell cycle arrest following treatment with several cytotoxic agents. By potentiating sustained arrest, USP42 stabilization of p53 may provide cells with the opportunity to repair damage, thereby likely promoting genome stability in normal cells (Hock et al. 2014). Aside from the above-mentioned USP family members, our lab has recently identified the OTU family member DUB, Otubain 1 (Otub1), as a novel p53-positive regulator (X.-X. Sun et al. 2011). In contrast to the above USPs, Otub1 regulates

p53 stability and activity primarily through a novel non-canonical mode that is independent of its DUB activity: suppression of the MDM2-cognate E2, UbcH5 (Figure 1-4). Consequently, overexpression of Otub1 induces p53-dependent apoptosis and inhibition of cell proliferation whereas knockdown of Otub1 attenuates p53 activation following DNA damage (X.-X. Sun et al. 2011). Consistently, Otub1 interacts with UbcH5. We have also shown that Otub1 is monoubiquitinated by UbcH5. Mechanistically, UbcH5 preferentially binds to monoubiquitinated Otub1. Mono-Ub interacts with UbcH5 through its backside on the donor ubiquitin-interacting surface. This interferes with the ability of UbcH5 to self-assemble into a ubiquitin charged conjugate which is a critical process in regulating ubiquitin transfer (Li et al. 2014). Thus, instead of targeting Otub1 directly, developing small molecule inhibitors to suppress UbcH5 activity based on the mechanism of Otub1 suppression could be another interesting strategy to reactivate p53. Recent structural studies of the E2-Otub1 complex would likely promote structure-based design of such E2 inhibitors (Juang et al. 2012; Sato et al. 2012; Wiener et al. 2012).

Taken together, these studies demonstrate the central role of DUBs in regulating p53-MDM2 signaling. From this body of work, we have learned that two of the keys factors regulating DUB specificity towards this signaling pathway include (1) a differential response based on the presence or absence of stress as well as the type of stress and (2) varying distributions across cellular compartments that regulate spatially defined pools of p53 and MDM2. DUBs that

localize to both the nucleus (USP2a and USP7) and the cytoplasm (USP10) have been identified.

Interestingly, the nucleolus, a hub for integrating stress responses, is known to house a diverse repertoire of proteins that function outside of ribosome biogenesis. While some of these proteins are known to regulate the cell-cycle and apoptosis, others serve to integrate extra-nucleolar stress signals into the regulation of processes classically associated with the nucleolus, such as those involved in ribosome biogenesis. Furthermore, there are close connections between p53-MDM2 signaling and the nucleolus. Interestingly however, in spite of this connection and the importance of ubiquitination in regulating the p53-MDM2 axis, no nucleolar DUB has been identified that regulates either protein. To address this, I have focused on the nucleolar DUB, USP36 that regulates important oncogenic pathways that intersect with ribosome biogenesis. I have found that USP36 interacts with both MDM2 and p53 in cells and in vitro. I show that USP36 deubiquitinates and stabilizes both proteins. These results reveal that USP36 is a novel nucleolar DUB that regulates both MDM2 and p53 and may play an important role in regulating p53 signaling. Future studies will tease apart the physiological relevance of its dual regulation on this pathway.

3.3 RESULTS

USP36 directly interacts with MDM2. To determine whether the p53-MDM2 pathway is regulated by nucleolar DUBs, we focused on the USP family member USP36, due to its known ability to integrate oncogenic signaling with ribosome biogenesis. Additionally, many of the nucleolar proteins (ARF and RPs) regulate this signaling pathway interact with MDM2. Therefore, We first tested whether USP36 interacted with MDM2. H1229 cells transfected with HA-MDM2 alone or together with Flag-UPS36 were subjected to co-IP using anti-Flag antibody. As shown Figure 3-1A, Flag-USP36 specifically co-immunoprecipitated with HA-MDM2 when both proteins were co-expressed. To determine whether endogenous USP36 interacts with MDM2, co-IP was performed using lysates from SJSA cells, which express relatively high levels of endogenous MDM2 (Dai et al. 2004). As shown in Figure 3-1C, endogenous MDM2 is co-immunoprecipitated with endogenous USP36 using anti-USP36 antibodies. This interaction appears to be weak possibly due to its transient nature, or an inefficiency of the antibody in co-IP assays.

Given the interaction we found in cells, and the fact that DUBs are often closely associated with the E3's they regulate, we wondered if MDM2 and USP36 directly interact. To test this question, we performed GST-pull down assays. We generated a GST-fused N-terminal USP36 fragment (aa 1-800) that

contains the USP36 domain and is fully proficient in catalyzing deubiquitination. This fragment was used in lieu of the full-length protein due to difficulties in

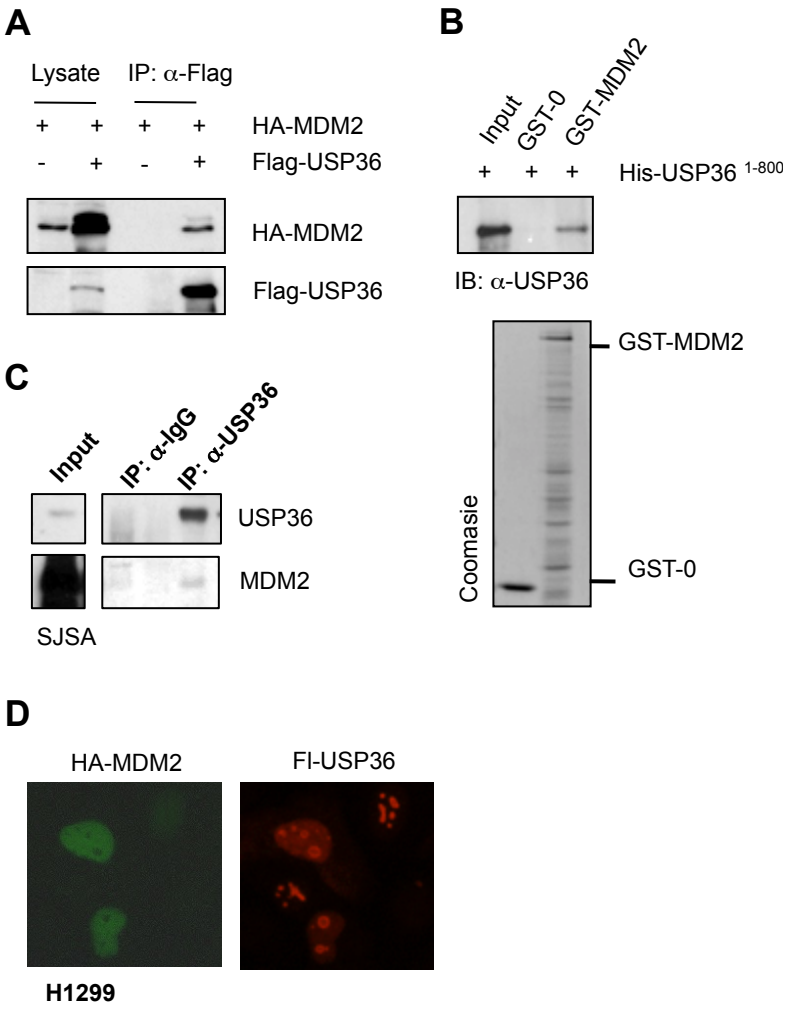


Figure 3-1: USP36 interacts with MDM2.

(A) Flag-USP36 and HA-MDM2 overexpressed in H1299 cells, followed by Flag IP. (B) USP36 interacts with MDM2 in vitro. His-USP36$^{1-800}$ and GST-MDM2 were incubated followed by GST pulldown. (C) Endogenous USP36 interacts with MDM2. (D) USP36 and MDM2 co-localize in nucleoplasm. Flag-USP36 and HA-MDM2 overexpressed in H1229 cells, followed by IF

purifying high quality full-length protein. Additionally, the USP36$^{1-800}$ fragment interacts with MDM2 in cells (Figure 3-2A). We incubated recombinant GST-USP36$^{1-800}$ or GST alone (GST-0) protein purified from bacteria and immobilized

on the glutathione-agarose beads with recombinant His-MDM2 also purified from bacterial. As shown in Figure 3-1C, His-MDM2 specifically bound to GST-USP36$^{1-800}$, but not GST-0, indicating that USP36 directly interacts with MDM2 in vitro.

MDM2 is known to localize to both the nucleus and the cytoplasm. Immunofluorescence (IF) staining typically shows diffuse nuclear staining, with exclusion from the nucleolus. Some nucleolar proteins such as Arf can relocalize MDM2 to the nucleolus (Weber et al. 1999). Although this observation has not been conclusively recapitulated in human cells, it is still an intriguing finding that led to question whether USP36 and MDM2 may interact through a similar mechanism. Furthermore, roughly 80% of USP36 within the nucleus is specifically localized to nucleoli (Figure 2-10). To test where USP36 interacts with MDM2, we performed IF staining with Flag-USP36 and HA-MDM2 in H1299 cells. As shown in Figure 2-1D, MDM2 still appears to be excluded from the nucleolus, following co-expression of USP36. In contrast, USP36 was found to be primarily nucleolar, and moderately nuclear when co-expressed with MDM2. This co-localization result suggests that USP36 most likely interacts with MDM2 in the nucleoplasm.

USP36 interacts with MDM2 through both its N-terminus and C-terminus. Next, we were interested in determining how USP36 interacts with MDM2. The

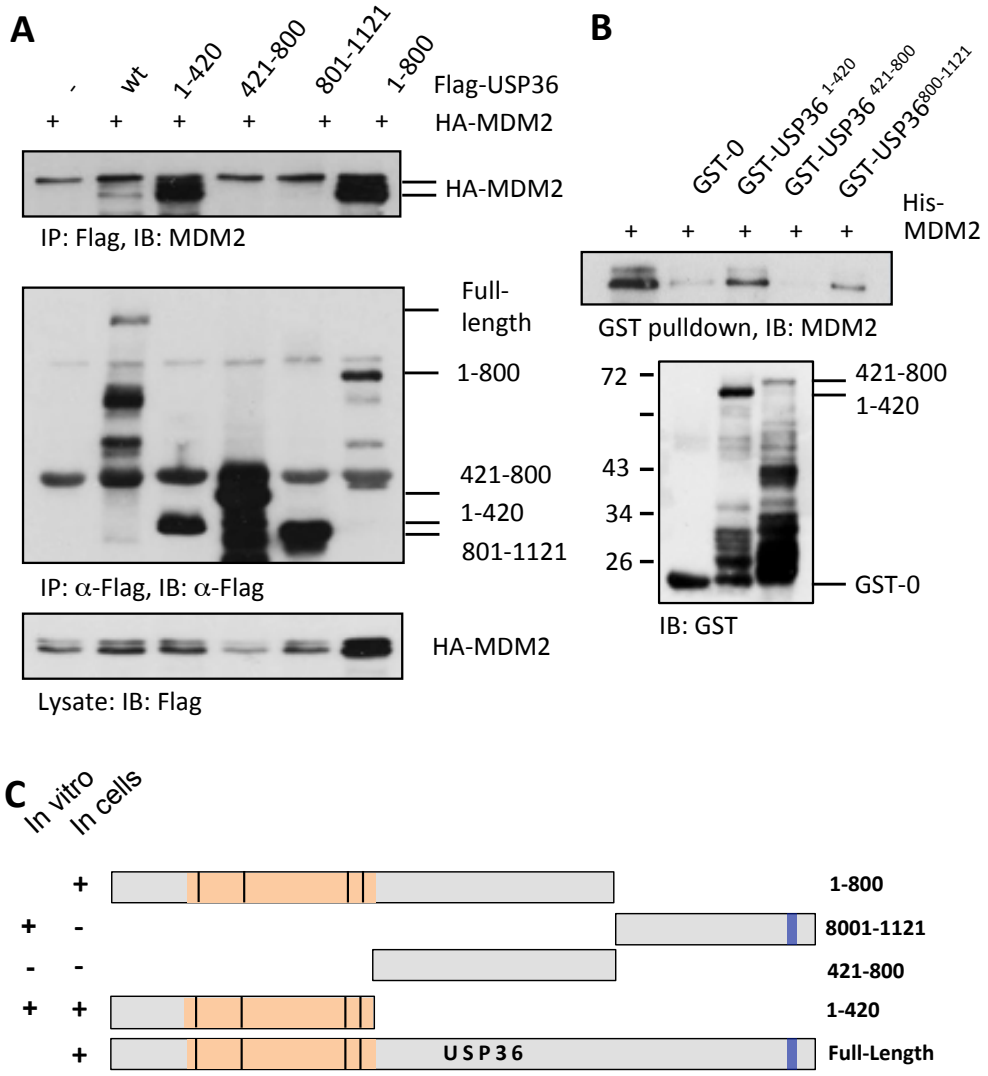


Figure 3-2: MDM2 interacts with the catalytic domain and C-terminus of USP36.

(A) MDM2 interacts with the catalytic domain of USP36 in cells. HA-MDM2 was co-transfected with Flag-USP36 fragments, followed by Flag IP. (B) MDM2 interacts with both the N-terminus and the C-terminus of USP36. GST-tagged USP36 fragments were incubated with His-MDM2, followed by GST pulldown. (C) Summary of mapping results both in cells and in vitro.

majority of USP36, outside of its catalytic domain, is intrinsically unstructured (Reed et al. 2015). Like USP36, its yeast ortholog, ubp10, has a high

concentration of Intrinsically disordered regions (IDRs) outside of the catalytic domain. In ubp10, these IDRs impart a scaffold-like property, as they are known facilitate protein-protein interactions (Reed et al. 2015). Regions such as these may be particularly relevant for DUBs to identify target substrates. Therefore, we hypothesized that similar regions within USP36 may also be enriched for protein-protein interactions and thus the site of MDM2 binding. Alternatively, if MDM2 were to interact with the catalytic domain this may suggest that USP36 has catalytic activity towards MDM2, as we have seen for c-Myc. To examine the site of binding, we first co-expressed HA-MDM2 with either Flag tagged full-length USP36 or its deletion mutants including Flag-USP36$^{1\text{-}420}$, Flag-USP36$^{421\text{-}800}$, Flag-USP36$^{801\text{-}1121}$, or Flag-USP36$^{1\text{-}800}$ and subjected cell lysates co-IP using anti-Flag antibody. As shown in Figure 3-2A, while full-length USP36 interacted with MDM2 as expected, we also observed an interaction between MDM2 and the N-terminal USP domain containing mutants, Flag-USP36$^{1\text{-}420}$ and Flag-USP36$^{1\text{-}800}$. Interestingly, we found that both nuclear localized fragments, lacking a nucleolar localization signal, interacted with MDM2 more strongly than the full-length USP36. This result is consistent with the previous observation that the two proteins co-localize in the nucleoplasm. Neither the middle region of USP36 (421-800) nor the far C-terminus (800-1121) were able to pull down MDM2. One technical issue with the above assay is that the 800-1121 fragment is known to localize to the nucleolus (Endo, Kitamura, et al. 2009). It is therefore unlikely, given the exclusion of MDM2 from the nucleolus, that we can assay this interaction in cells. Instead we choose to employ an in vitro interaction assay.

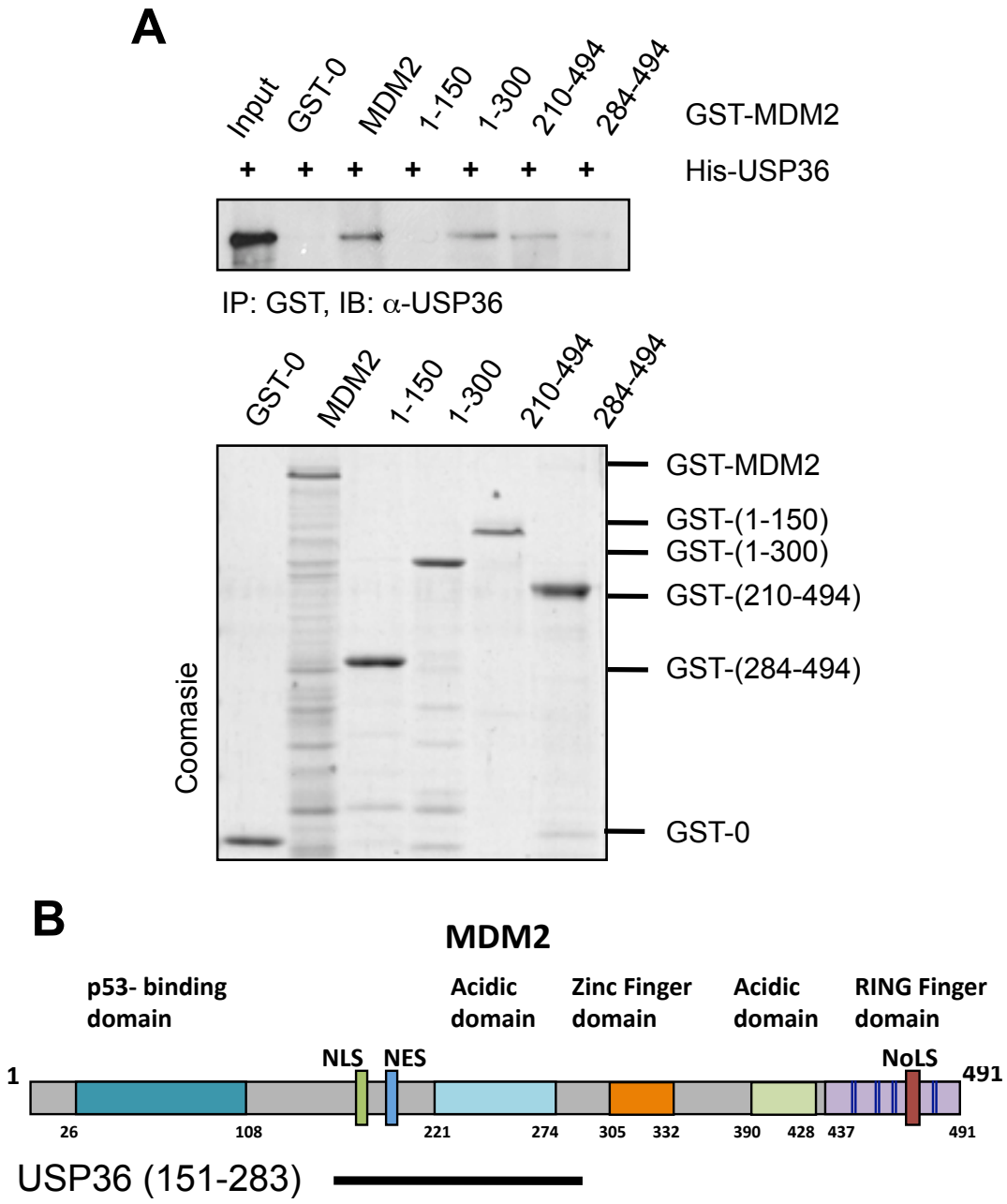


Figure 3-3: : USP36 interacts with the central acidic domain of MDM2.

(A) In vtiro mapping of USP36 to MDM2. GST-tagged fragments of MDM2 were incubated with His-USP36 (1-800), followed by GST pulldown. (B) Schematic of MDM2 protein with indicated USP36 binding site.

Using bacterially purified GST-tagged versions of the truncated mutants 1-420, 421-800 and 801-1121, we incubated these fragments with His-MDM2 and carried out GST pull downs. Figure 3-1B shows that as expected we again have interaction between MDM2 and the catalytic domain of USP36. Confirming our in cell co-IP assays, we again did not detect an interaction within the middle region. However, surprisingly, we found MDM2 also interacts with the far C-terminal fragment, thus confirming that localization differences preclude an interaction between this region and MDM2 Figure 3-2C,D. Taken together, we have found that MDM2 interacts with both catalytic domain of USP36 as well its far C-terminus, containing the nucleolar localization signal as summarized in Figure 3-2C. Whether these interactions are mutually exclusive remains unknown. However, we can conclude that the C-terminus of USP36 is sufficient for the two proteins to interact in cells.

USP36 binds to the central acidic domain of MDM2. In order to examine the reciprocal mapping of USP36 to MDM2, we carried out a similar in vitro GST-pull-down assays by incubating recombinant GST-MDM2 or its various deletion mutants with recombinant His-USP36$^{1-800}$. Figure 3-3A shows the results of our GST pulldown. Again as expected, USP36$^{1-800}$ specifically bound to full length GST-MDM2, but not GST-0 (Figure 3-3A). In addition, USP36 interacted with

GST-MDM2$^{1-300}$ and GST-MDM2$^{210-494}$, but not the N-terminal mutant GST-MDM2$^{1-150}$ or the C-terminal mutant GST-MDM2$^{284-494}$. The identified binding region, 151-284, shown in Figure 3-3B includes all of the central acidic domain, the nuclear localization signal and the nuclear export signal. Thus, we conclude that USP36 interacts with the central acidic domain of MDM2 (Figure 3-3B).

USP36 stabilizes MDM2 resulting in increased steady state levels. As USP36 is a deubiquitinating enzyme, we predicted that it might regulate MDM2 stability. Indeed we had observed that MDM2 levels are increased when co-expressed with USP36 Figure 3-1A (compare the second lane to the first lane). Therefore, we sought to further examine the effect of USP36 on MDM2 levels. As shown in Figure 3-4A with increasing amounts of Flag-USP36 transfected into H1299 cells, the levels of co-transfected HA-MDM2 were also increased in a dose-dependent manner. To examine whether this effect is dependent on the catalytic activity of USP36, we transfected H1299 cells with HA-MDM2 alone or together with wild-type USP36 or its catalytic-inactive C131A mutant. IB analysis showed that unlike the wild-type USP36, the C131A mutant failed to induce the levels of MDM2, indicating that the DUB activity of USP36 is required for its role in stabilizing MDM2 Figure 3-4B. To look at whether USP36 could stabilize endogenous MDM2, we constructed doxycycline inducible Flag-USP36 cell lines in the background of U2OS cells. Figure 3-4C shows a time course study for two different clones. Consistent with above results, increasing UPS36 levels by exposing cells to dox for increasing times also resulted in increasing levels of MDM2. Furthermore, siRNA-mediated knockdown of USP36 reduced the levels

of endogenous USP36 in SJSA cells (Figure 3-4D). Thus endogenous USP36 also regulates endogenous MDM2 levels.

To further test whether the induction of MDM2 by USP36 is due to the stabilization of MDM2, we performed protein half-life assays. H1299 cells were transfected with HA-MDM2 alone or together with Flag-USP36, followed by treatment with the translational inhibitor cyclohexamide (CHX) for different times overexpression of USP36 markedly prolonged the half-life of transfected MDM2 in cells (Figure 3-5A, B). In the absence of USP36, the half-life of exogenous MDM2 was 35 min, whereas in the presence of overexpressed USP36, the half-life of exogenous MDM2 increased well over 90 minutes (Figure 3-5A, B). To look at whether endogenous USP36 affects the half-life endogenous MDM2, we transfected SJSA cells with control scrambled or USP35 siRNA followed by similar half-life assays as above. As shown in Figure 3-6AB, knockdown of USP36 reduced the half-life of endogenous MDM2 in SJSA cells. Under scrambled transfected conditions the half-life of endogenous MDM2 was approximately 20 minutes and this was reduced by roughly half to 8 minutes when USP36 was knocked down (Figure 3-6B). Together, our data demonstrate that USP36 plays a role in stabilizing MDM2 in cells.

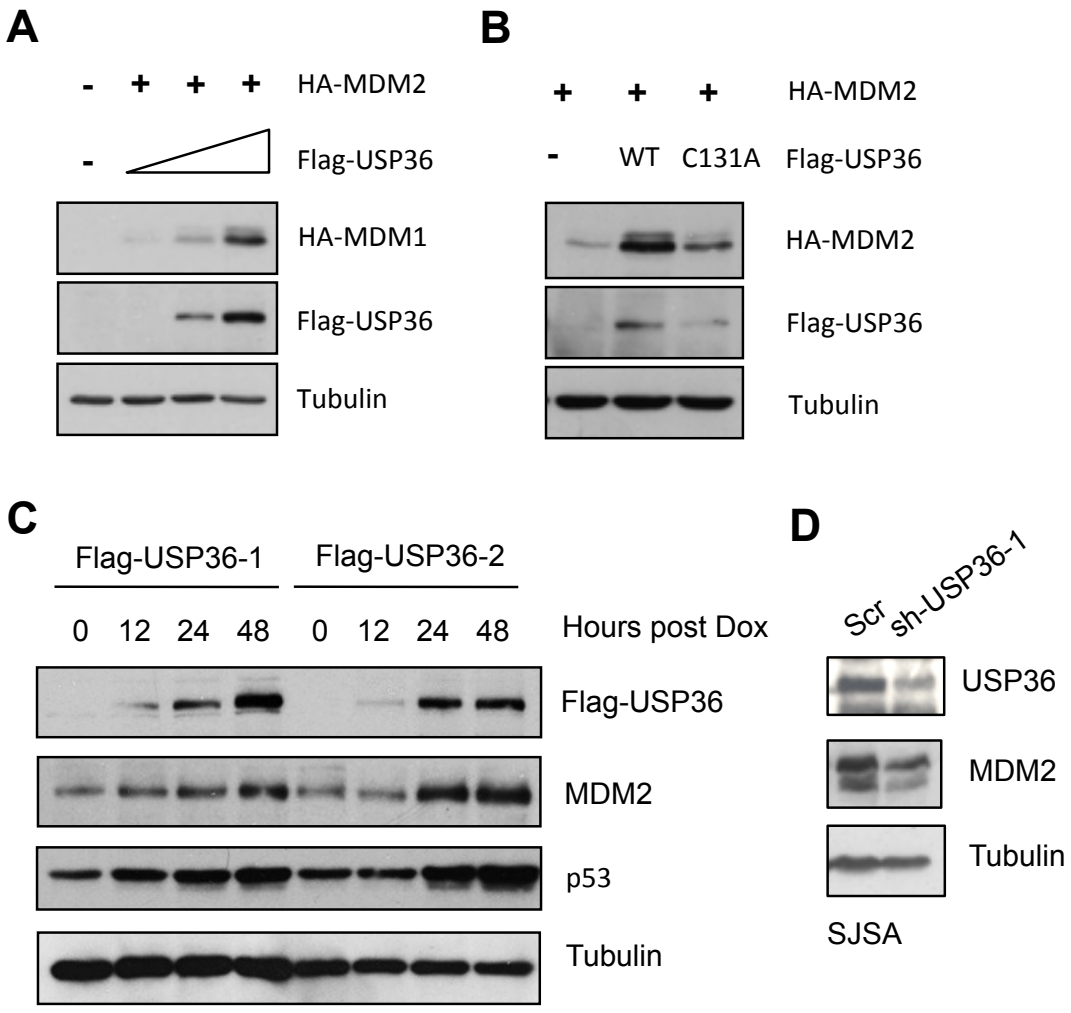


Figure 3-4: USP36 stabilizes MDM2.

(A) Overexpressed USP36 stabilizes MDM2. Increasing amounts of Flag-USP36 were co-transfected with HA-MDM2. (B) USP36^{C131A} fails to stabilize MDM2. Flag-USP36 and Flag-USP36^{C131A} were co-transfected with HA-MDM2. (C) USP36 stabilizes MDM2 in a dose-dependent manner. Two doxycycline inducible Flag-USP36 clones were used to do a time course followed by western blot to assay for MDM2 levels. (D) Depletion of USP36 destabilizes MDM2. SJSA cells were transfected with either scramble or siRNA targeting USP36 for 48 hours.

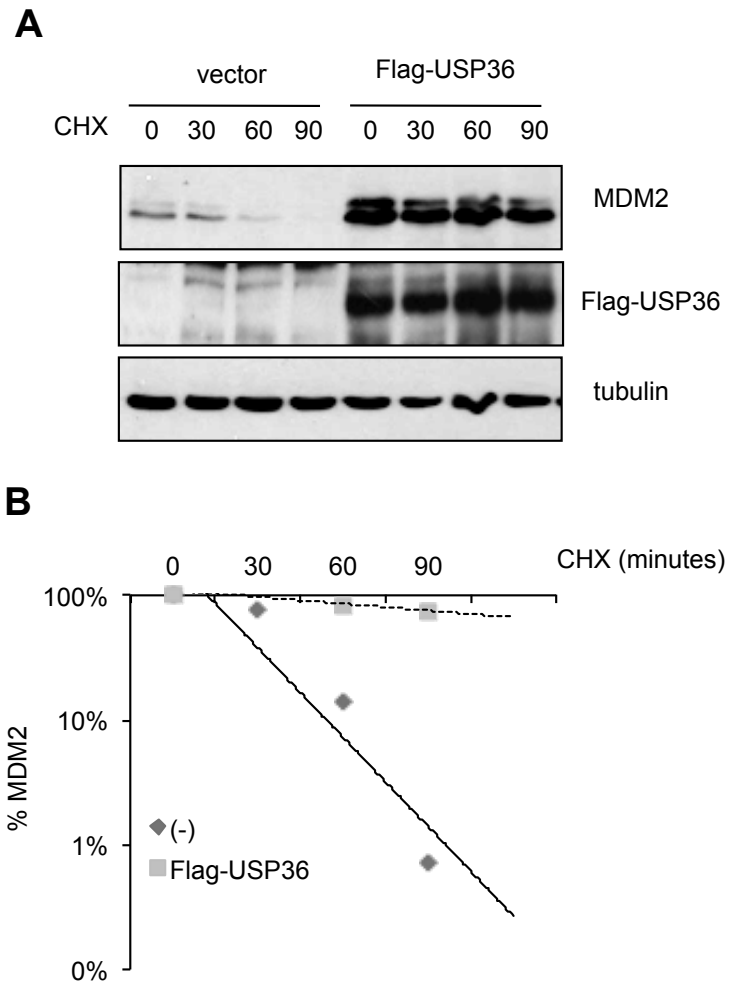


Figure 3-5: USP36 increases the half-life of MDM2.

(A) Flag-USP36 was co-transfected with HA-MDM2 into H1299 cells. Cells were then treated with cyclohexamide (CHX) for 0, 30, 60, or 90 minutes. MDM2 was then assayed by western blot. (B) The half-life of MDM2 following co-transfection with an empty vector in H1299 cells is 30 min. When Flag-USP36 is co-transfected with MDM2, the half life is well beyond 90 minutes.

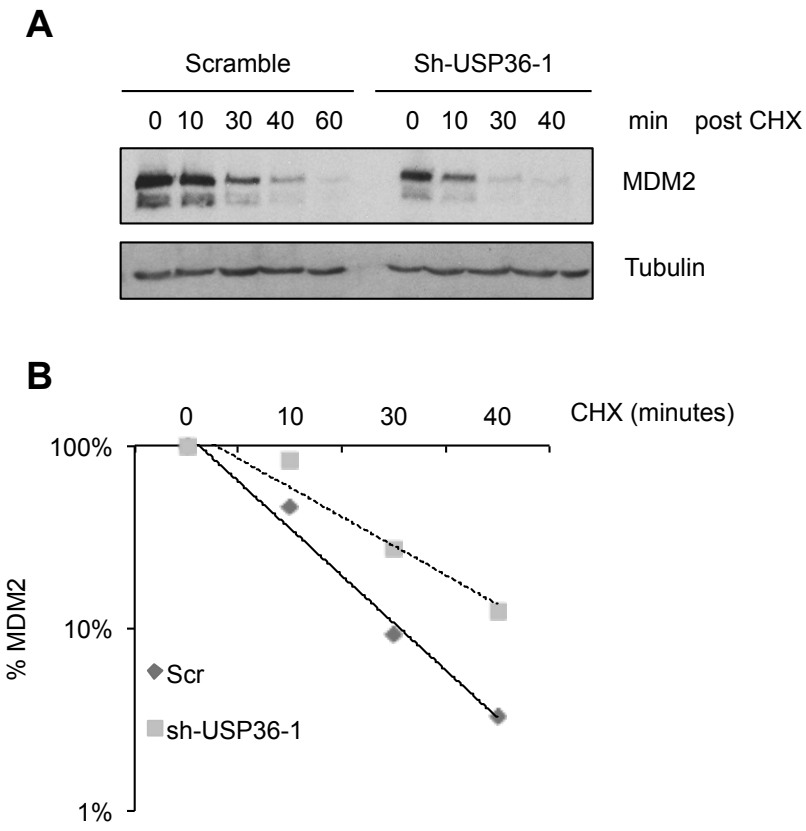


Figure 3-6: Depletion of endogenous USP36 rapidly decreases MDM2.

(A) SJSA cells were infected with a lentivirus carrying either shRNA against a scramble control or USP36 for 48 hours. Cells were then treated with cyclohexamide for either 0, 10, 30, or 40 minutes and assayed by western blot. (B) The values relative to tubulin are plotted below and the half-life determined. In scramble-infected cells MDM2's half-life was 20 min vs. 8 min in sh-USP36 infected cells.

USP36 deubiquitinates MDM2. The above observation that USP36 stabilizes MDM2 and that this is dependent on its catalytic activity suggested that USP36 may be a deubiquitinase for MDM2. To address this question, we carried out in vivo ubiquitination assays. In this experiment, we transfected H1299 cells with HA-MDM2 and His-Ubiquitin presence of either Flag-USP36 or the

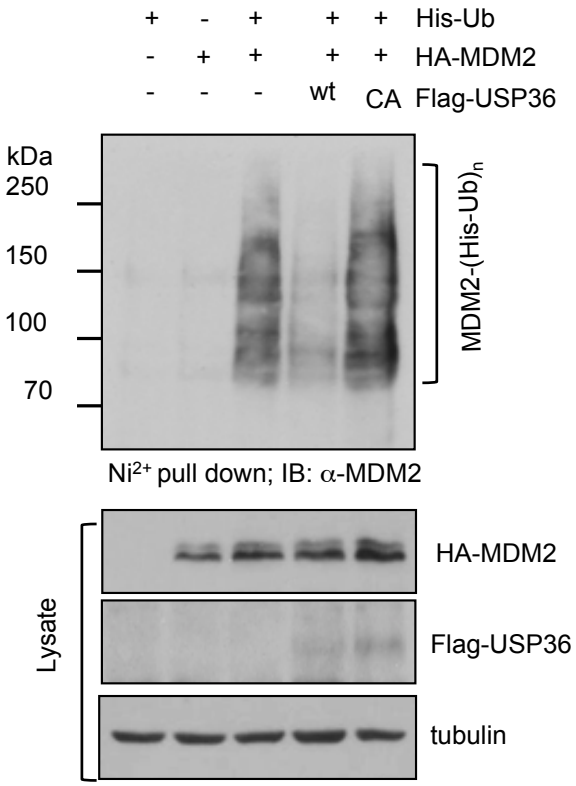


Figure 3-7: USP36 deubiquitinates MDM2.

H1299 cells were transfected with His-Ub, HA-MDM2, and either Flag-USP36 or Flag-USP36^{C131A}. 48 hours post transfection cells were treated with MG132 for 6 hours and harvested. Lysates were then subject to Ni-NTA pulldown and assayed for MDM2 poly-ubiquitinated species by western blot.

Flag-USP36^{C131A} mutant. We then pulled down all poly-ubiquitinated species and specifically assayed by western blot for those that were conjugated to MDM2. Figure 3-7 shows that the expression of wild type USP36, but USP36^{C131A}, significantly reduced the polyubiquitinated species of MDM2. Furthermore, the C131A mutant increases poly-ubiquitinated MDM2 as compared to empty vector transfection, suggesting a dominant negative role for this mutant in deubiquitinating MDM2.

USP36 stabilizes p53. Next, we asked whether USP36 also regulates p53 stability, as MDM2 is a master negative regulator of p53. To test this, we co-expressed different combinations of p53 and MDM2, in the absence or presence of either wild-type or catalytically inactive USP36 (Figure 3-8, Figure 3-4C). As expected when p53 is co-expressed with MDM2, its levels decrease dramatically. However, instead of finding a further decrease in p53, upon co-expression of wild-type USP36, we found that it partially rescued p53 levels. Interestingly, expression of a USP36 mutant lacking the NoLS ($USP36^{\Delta BM5}$) stabilized both MDM2 and p53 to a greater extent than the wild-type USP36, supporting our hypothesis that USP36 interacts with MDM2 in the nucleoplasm (Endo, Kitamura, et al. 2009). This result suggested that USP36 may deubiquitinate p53.

USP36 deubiquitinates p53. Next we directly tested whether USP36 was able to deubiquitinate p53. To do so, we conducted an in vivo ubiquitination assay in H1299 cells using the Ni^{2+}-NTA purification method (Borsig et al. 1997). H1299 cells were transfected with different combinations of His-ubiquitin, p53, HA-MDM2 with either Flag-tagged wild-type USP36, its C131A mutant, or USP7 (a positive control) (Li et al. 2002). Right before harvesting, we treated cells with the

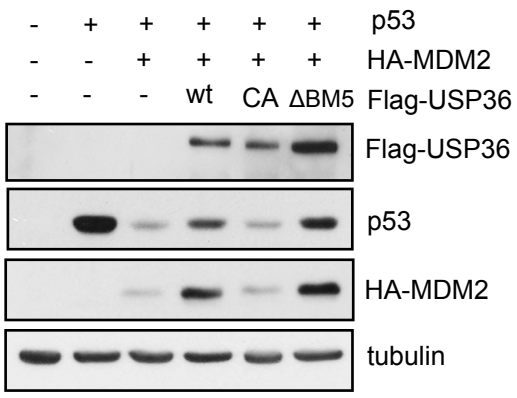


Figure 3-8: USP36 partially rescues MDM2-mediated degradation of p53.

H1299 cells were transfected with p53, HA-MDM2, Flag-USP36 or Flag-USP36^{C131A}, harvested 48 hours later and assayed by western blot.

proteasome inhibitor MG132 so that we could readily detect poly-ubiquitinated species. As shown p53 poly-ubiquitination was increased with the addition of MDM2 and abolished following co-expression of USP7 (compare lane 6 to lane 3, top panel of Figure 3-9A.) Overexpressing the wild-type form of USP36, and not its catalytically inactive mutant, completely abolished MDM2-mediated p53 polyubiquitination (compare lanes 4 and 5 to lane 3, respectively, top panel of (Figure 3-9A). Using a co-IP method, in which we pulled down V5-Ub, we found similar results (Figure 3-9B). Overall, These results clearly show that USP36 deubiquitinates p53 in cellsas the reduction of p53 ubiquitination requires the catalytic activity of USP36.

USP36 also interacts with p53. Given that USP36 deubiquitinates p53, we asked whether the two proteins interact. H1229 cells were transfected with p53 alone or together with Flag-USP36 and subject to co-IP using anti-Flag antibody.

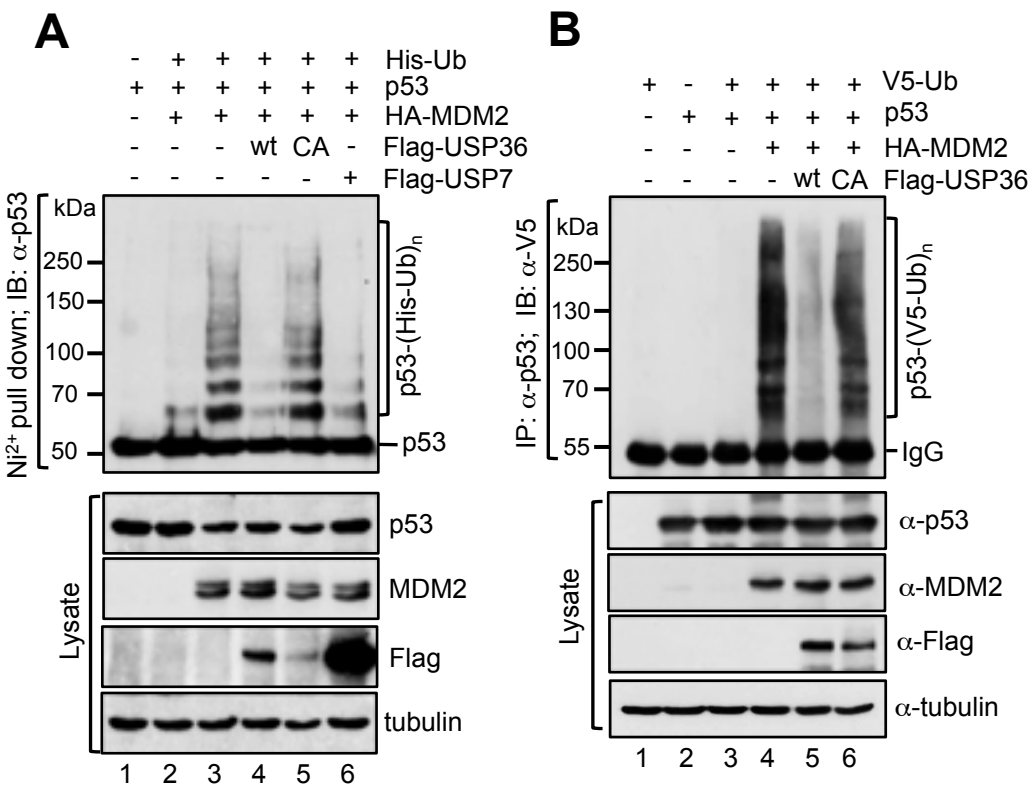


Figure 3-9: USP36 deubiquitinates p53.

(A) H1299 cells were transfected with His-ubiquitin, p53, MDM2, Flag-USP36 wild type and Flag-USP36^{C131A} or the positive control USP7. 6 hours prior to harvesting cell were treated with MG132, then processed for Ni-NTA pulldown. The protein bound beads were then assayed by immunoblot for p53 and the indicated antibodies were used to detect transfected proteins in the lysate. (B) V5-Ub was transfected with combinations of p53, HA-MDM2, Flag-USP36 and Flag-USP36^{C131A}. This was followed by p53 immunoprecipitation and detection of V5-Ub.

As shown in Figure 3-10A, p53 was specifically co-immunoprecipitated with Flag-USP36. To test if this interaction was direct, GST-pulldown assays were conducted. As Figure 3-10B, purified His-USP36 was pulled down by GST-p53, but not GST alone. Thus, these data reveal that USP36 interacts with p53 in cells

and in vitro. Given our observation that wild type USP36 can only partially rescue p53

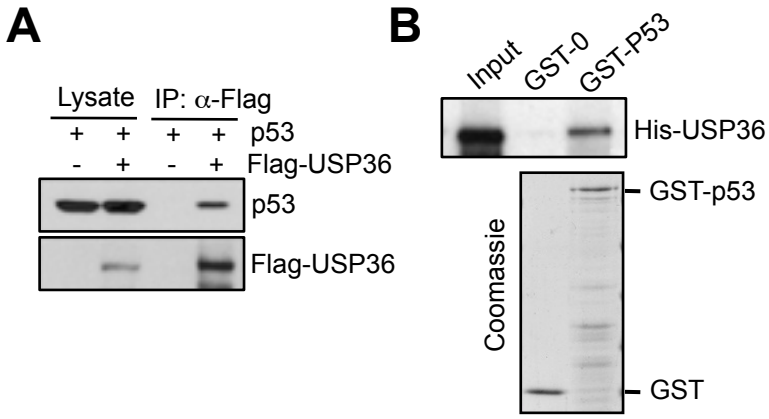


Figure 3-10: USP36 interact with p53.

(A) USP36 interacts with p53 in cells. H1299 cells were co-transfected with p53 and Flag-USP36, followed by Flag pull-down. (B) USP36 directly interacts with p53. Bacterially purified His-USP36 (1-800) and GST-p53 were incubated, followed by GST pull -own.

degradation, this raises a question as to whether USP36 regulates p53 and MDM2 separately.

3.4 DISCUSSION

In this chapter, we show that USP36 interacts directly with both MDM2 and p53, deubiquitinates and stabilizes both proteins in cells. Consequently, overexpression of USP36 induced the levels of both MDM2 and p53. The catalytically inactive mutant USP36^{C131A} failed to deubiquitinate both MDM2 and p53 and thus failed to induce their levels. These results demonstrate that USP36 is a novel deubiquitinase for both MDM2 and p53 that controls the stability of both proteins. Future in vitro deubiquitination assays using recombinant proteins would further support this notion.

USP36, like USP7, deubiquitinates and stabilizes both p53 and MDM2.

DUBs have been shown to play a complex role in regulating the MDM2-p53 pathway. Some DUBs specifically regulate either p53 or MDM2, but not both. For example, USP2a deubiquitinates and stabilizes MDM2 and MDMX, while not effecting polyubiquitination of p53. This results in increased MDM2/MDMX-mediated degradation of p53 (Stevenson et al. 2007). USP15 appears to regulate MDM2 in a similar manner in cancer cells, as knockdown of USP15 decreases MDM2 (Zou et al. 2014). As a result, p53 increases and apoptosis is induced. On the other hand, USP10 has been shown to only deubiquitinate p53, but not MDM2 (Yuan et al. 2010). Our data indicate that USP36, like USP7 deubiquitinates and stabilizes both MDM2 and p53 (Li et al. 2002; Li et al. 2004; Meulmeester et al. 2005). We have identified several similarities and differences

between the way in which USP7 and USP36 regulate the p53-MDM2 pathway. One similarity between USP36 and USP7 is that both DUBs interact directly with both MDM2 and p53. In vitro assays suggest that p53 and MDM2 compete for the same binding site in USP7 (Hu et al. 2006). MDM2 outcompetes p53 for this interaction even when p53 levels are 10-fold higher. The binding of p53 and USP7 to MDM2 is not mutually exclusive. Therefore, the formation of USP7-MDM2-p53 is possible. Whether USP7 regulates the complex, as a whole, is still unclear. However, there is sufficient evidence to conclude that under non-stressed conditions endogenous USP7 likely prefers MDM2, whereas upon DNA damage the high affinity interaction with MDM2 decreases, most due to phosphorylation of MDM2 that precludes USP7 binding (Li et al. 2004; Meulmeester et al. 2005) . This shifts USP7's preference toward interacting with and stabilizing p53. Given that USP36 directly interacts with both p53 and MDM2, future work should address its affinity to each target, perhaps in vitro as well as under stress conditions.

While there is clearly overlap between USP36 and USP7 in terms of their interactions with p53 and MDM2, they also differ in a number of respects. First, although overexpression of USP36 induces the levels of both p53 and MDM2, similar to USP7, this does not result in dramatic induction of p53 mediated arrest or apoptosis as it does in the case of USP7 (Li et al. 2002). In fact, in spite of observing relatively dramatic increases in p53, I have not been able to detect robust induction of canonical p53 target genes (Data not shown). A second way in which USP36 and USP7 differ is that knockdown of USP36 results in

decreased levels of both p53 and MDM2. This is contrasted with robust and reproducible induction of p53 following knockdown of USP7 (Meulmeester et al. 2005). Functionally, cells undergo p53-mediated apoptosis. While, there is an observation made in normal human fibroblasts that partial knockdown of USP7 destabilizes p53 (while complete knockdown induces it), it is not clear what the relevance of this finding is (Meulmeester et al. 2005). Furthermore, USP7 inhibitors that may one day be used in the clinic, robustly activate p53 (Nicholson & Suresh Kumar 2011; Menard & Sulea 2012; Colland et al. 2009). However, if partial inhibition of USP7 were confirmed to destabilized p53, this would clearly need to be explored in more detail as it could challenge the clinical efficacy of this target. In any case, the dual decrease in both MDM2 and p53 (Data not shown) upon knockdown of USP36 needs to be further explored for its physiological relevance. It would be interesting to examine whether there are differential effects based on the degree of USP36 knockdown. However, as mentioned for USP7, this would complicate the rationale for targeting USP36. Differential effects on the MDM2-p53 pathway could have unwanted, even dangerous effects, as it would be very difficult to predict levels of inhibition across tissues in a clinical setting.

USP36 may regulate the MDM2-p53 axis in the nucleoplasm.

Several pieces of evidence suggest that USP36, as a primarily nucleolar localized DUB, may regulate the MDM2-p53 pathway in the nucleoplasm. First, our IF data showed that USP36 did not re-localize MDM2 from the nucleoplasm into the nucleolus; instead, the two proteins co-localize in the nucleus **(**Figure

2-10). Second, the nucleolar localization-defective mutant USP36$^{\Delta BM5}$, which lacks a C-terminal NoLS and localizes to the nucleoplasm, stabilized MDM2 and partially abolished MDM2-mediated p53 degradation, to a slightly greater extent than the wild-type USP36 (Figure 3-6). These results imply that at steady-state levels, a small pool of nucleoplasmic USP36 can bind to and regulate MDM2 and p53 stability, while the majority of USP36, which is nucleolar, does not associate with MDM2 or p53. Two possible scenarios could explain this observation. First, like NPM, USP36 may shuttle between the nucleoplasm and the nucleolus. Determining the factors that regulate this shuttling may shed light on how USP36 regulates p53/MDM2 signaling. Alternatively, and not necessarily mutually exclusive, USP36 may deubiquitinate MDM2 and p53 within the nucleolus. The deubiquitinated forms of both protein may then be translocated back to the nucleoplasm, similar to the way in which USP36 regulates c-Myc (Sun et al. 2015). In this scenario, we may fail to observe an accumulation of MDM2/p53 within in the nucleolus due to a very transient presence there.

Interestingly, the nucleolar localization of USP36 is intimately tied to Nucleophosmin (NPM) as it has been shown that knockdown of NPM can almost entirely relocalize USP36 to the nucleoplasm. Although NPM is strongly targeted to the nucleolus, it was shown by FRAP to traffic between other nucleoli and the nucleoplasm. The dynamic behavior of NPM, and nucleolar proteins more generally, suggests that the “nuclear pool” of USP36 is not static. Instead, we hypothesize it may undergo routine exchange with the nucleolus.

The flux of proteins between the nucleus and the nucleolus is an integral feature of cellular stress responses. Arguably, one of the strongest links between these two compartments is the p53-MDM2-MDMX signaling axis. Both MDM2 and p53 can traffic through and the nucleolus as well as interact with proteins of this compartment under various conditions. MDM2, for example, may be sequestered in the nucleolus by the tumor suppressor p19 ARF in response to oncogenic stress (Weber 1999). Similarly, PML can recruit MDM2 to the nucleolus in response to DNA damage (Bernardi et al. 2004). Conversely, following disruption of rRNA synthesis, ribosomal proteins (RPs) undergoing assembly into ribosome subunits within nucleoli are redistributed into the nucleus, where they interact with MDM2. Given that we have substantial evidence that USP36 can regulate nuclear p53 and MDM2, it will be interesting to thoroughly examine this relationship under conditions such as ribosomal stress, in which nucleolar components are redistributed.

The Role of USP36 in regulating p53 activity.

We show that overexpression of USP36 stabilizes both MDM2 and p53. Yet how USP36 regulates the activity of both proteins is still not clear. Our mapping results showed that USP36 interacts with a region in MDM2 that encompasses the central acidic domain, as well the nuclear localization signal and the nuclear export signal. Other nucleolar proteins such as B23 Nucleolin, Nucleostemin, multiple ribosomal proteins, as well as ARF that regulate p53 levels also interact with MDM2 through this region (Saxena et al. 2006; Kurki et al. 2004; Zhang & Lu 2009; Honda & Yasuda 1999). Mechanistically, these

nucleolar proteins bind the central acidic and/or Zinc finger domains, leading to inhibition of MDM2 E3 activity towards p53 (Cheng et al. 2014). While our mapping indicates an overlap in binding, it is unlikely that the mechanism of MDM2 inhibition is similar to nucleolar proteins. This is primarily because the effect of USP36 on both p53 and MDM2 is dependent on its catalytic activity.

Through their ability to interact with and inhibit MDM2, the above mentioned nucleolar proteins all promote p53 induction of cell cycle arrest, suggesting they possess intrinsic tumor suppressor function. Indeed, mice heterozygous for NPM have a predisposition for myeloid and lymphoid leukemia (Sportoletti et al. 2008). Whether, this effect is p53 dependent remains unknown. As USP36 is also known to regulate proteins involved in ribosome biogenesis, it is intriguing to speculate that it may also function as a haploinsufficient tumor suppresser.

Overall, we have identified USP36 as novel regulator the p53-MDM2 pathway. While not fully understood at this time, it is clear that its regulation of this signaling axis is unique when compared to other DUBs, such as USP7. Understanding the physiological relevance of USP36 on this pathway is the obvious next step. We and others have shown key evidence that USP36 is likely oncogenic including (1) its overexpression in cancers (2) its positive regulation of ribosome biogenesis (3) its stabilization of the oncoprotein c-Myc and (4) its ability to repress p21 through deubiquitination of histone 2b. Understanding how USP36 regulates the critical p53-MDM2 tumor suppressor pathway should be a primary goal in pursuing it as an oncogenic target.

4 Materials and Methods

4.1 Materials

	Functonal WT p53	Non-functional p53 or null	Source
H1299		✓	Non-small cell lung carcinoma
Hela		✓	Cervical carcinoma
HCT116 (-/-)		✓	Colorectal carcinoma
293		✓	Embryonic kidney
HCT116 (+/+)	✓		Colorectal carcinoma
RKO	✓		Colorectal carcinoma
SJSA	✓		Osteosarcoma
U2OS	✓		Osteosarcoma
WI38	✓		Lung fibroblast

Table 2: Cell Lines

AB	Source	Details
α-USP36	Endo et al	polyclonal
α- SP1	Abcam	Monoclonal
α- B23	Zymed	Monoclonal
α- H2b	Millipore	Polyclonal
α- H2bub1	Millipore	Monoclona l(61)
α- Flag	Sigma	Moncolonal (M2)
α- Tubulin	Sigma	Monocolonal
α- His	Pierce	Monoclonal
α- GST	Pierce	Monoclonal (HIS.H8)
α- V5	Invitrogen	Monoclonal (2F11F7)
α- UBF	Santa-Cruz	Monoclonal (F9)
α- p21	Pierce	Monoclonal (CP74)
α- MDM2	Santa-Cruz	Monoclonal (SMP14)
α- HA	Santa-Cruz	Monoclonal (12CA5)
α- p53	Santa-Cruz	Monoclonal (DO-1)

Table 3: List of Antibodies

Primer	Forward	Reverse
p21waf1/cip1	5′-ATGTCAGAACCGGCTGGGGATG-3′	5′-TTAGGGCTTCCTCTTGGAGAAG-3′;
MDM2	5′-TCAAGGTGACACCTGTTCTC-3′;	5′-AACCACCTCACAGATTCCAG-3′
GAPDH	5′-CCACCCATGGCAAATTCCATGGCA-3′	5'-TCTAGACGGCAGGTCAGGTCCACC-3'
Bax1	5′-TTCATCCAGGATCGAGCAGG-3′	5′-AGGAAGTCCAATGTCCAGCC-3′
PUMA	5'-ACCTCAACGCACAGTACGAG-3'	5'-CCCATGATGAGATTGTACAGGA -3'
p21 (UP)	5'-AGCAGGCTGTGGCTCTGATT-3'	5'-CAAAATAGCCACCAGCCTCTTCT -3'
p21 (Start)	5'-AGCCGGAGTGGAAGCAGA-3'	5'-AGTGATGAGTCAGTTTCCTGCAAG-3'
p21 (Mid)	5'-CCAGGGCTGCGATTAGGAA-3'	5'-GTGTCCCTCATGGGTGTGAAT-3'
p21(End)	5'-CCTCCCACAATGCTGAATATACAG-3'	5'-AGTCACTAAGAATCATTTATTGAGCACC-3'

Table 4: List of primer sequences

Buffer	Components
NP40	50 mM Tris/HCl [pH 8.0], 0.5% Nonidet P-40 (NP-40), 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 0.25 µg/ml
Buffer I	6 M guanidinium-HCl, 0.1 M Na2HPO4/NaH2PO4, 10 mM Tris-HCl [pH 8.0], 10 mM β-mercaptoethanol
Buffer II	8 M urea, 0.1 M Na2HPO4/NaH2PO4, 10 mM Tris-HCl [pH 8.0], 10 mM β-mercaptoethanol
Buffer III	8 M urea, 0.1 M Na2HPO4/NaH2PO4, 10 mM Tris-HCl [pH 6.3], 10 mM β-mercaptoethanol
Buffer IV	200 mM imidazole, 0.15 M Tris-HCl [pH 6.7], 30% glycerol, 0.72 M β-mercaptoethanol, and 5% SDS
Buffer A	10mM HEPES [pH 7.9], 1.5mM MgCl2, 10mM KCl, 0.5 mM DTT
S1	0.25 M Sucrose, 10 mM MgCl2
S2	0.35 M Sucrose, 0.5 mM MgCl2
S3	0.88 M Sucrose, 0.5 mM MgCl2
RIPA	50 mM Tris, [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5 % Deoxycholate,
TEB	PBS containing 0.5% Triton X 100 (v/v), 2mM phenylmethylsulfonyl fluoride (PMSF), 0.02% (w/v) NaN3
BC100	20 mM Tris, pH 8, 0.1 M KCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, aprotinin, leupeptin, and pepstatin, 1ug/ml each
SNNTE	50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% Nonidet P-40, 500 mM NaCl, and 5% sucrose.
Buffer AA	10mM HEPES [pH 7.9], 1.5mM MgCl2, 10mM KCl, 0.5 mM DTT, 0.34M Sucrose, 10% glycerol, 0.05% Triton-X.
Buffer AAA	10mM HEPES [pH 7.9], 1.5mM MgCl2, 10mM KCl, 0.5 mM DTT, 0.34M Sucrose, 10% glycerol
Solution B	3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, protease inhibitors
SA-β-Gal stain solution	40mM citric acid/ Na phosphate buffer (ph 6.0), 5mM $K_4[Fe(CN)_6]3H_2O$, 5mM $K_3[Fe(CN)_6]$, 150mM sodium chloride, 2mM Magnesium chloride, 1mg/ml^{-1} X-gal in distilled water
TBST	50 mM Tris-Cl, [pH 7.6], 150 mM NaC, Tween 20
EDU staining buffer	(100mM Tris, 4mM CuSO4, 5uM azide dye (Click Chemisty Tools), 100mM ascorbic acid (*added last) in PBS)

Table 5: List of buffers

USP36 plasmids	Source
Flag-USP36	Endo et. al. JCS 2009, Endo et. al. JCS 2009
Flag-USP36^{C131A}	Endo et. al. JCS 2009, Endo et. al. JCS 2009
Flag-USP36 $^{1-420}$	Sun et. al. PNAS 2015
Flag-USP36 $^{421-800}$	Sun et. al. PNAS 2015
Flag-USP36 $^{1-800}$	Sun et. al. PNAS 2015
Flag-USP36 $^{800-1121}$	Sun et. al. PNAS 2015
Flag-USP36 ^{BM5}	Sun et. al. PNAS 2015
His-USP36 $^{1-800}$	subcloned from Flag-USP36 into pPROEX-HT vector (Invitrogen)
His-USP36 $^{1-800\ C131A}$	subcloned from Flag-USP36 into pPROEX-HT vector (Invitrogen)
GST-USP36 $^{1-800}$	subcloned from Flag-USP36 into pGEX.4T.1 vector (Pharmacia)
GFP-USP36	subcloned from Flag-USP36 into pEGFP-N1 vector (Clonetech)
V5-USP36	Sun et. al. PNAS 2015
MDM2 plasmids	**Source**
HA-MDM2	Dai and Lu MCB 2004
GST-MDM2	Jin et. al. JBC 2002
GST-MDM2 $^{1-150}$	Jin et. al. JBC 2002
GST-MDM2 $^{1-300}$	Jin et. al. JBC 2002
GST-MDM2 $^{210-494}$	Jin et. al. JBC 2002
GST-MDM2 $^{284-494}$	Jin et. al. JBC 2002
His-MDM2	Dai and Lu MCB 2004
Other plasmids	**Source**
p53	Dai and Lu JBC 2004
His-UB	Dai and Lu JBC 2004
Flag-H2b	Mushui Dai
Flag-RNF20	Blank et. al. Nature medicine 2012

Table 6: List of Plasmids

Name	Sequence	Seq targeted (aa)
shRN- 1	5′-CGTCCGTATATGTCCCAGAAT-3′	351-378
shRNA-2	5′-GCGGTCAGTCAGGATGCTATT-3′	1049-1057

Table 7: shRNA sequences.

4.2 Methods

Cell Culture. All cell lines except W138 grown in DMEM supplemented with 10% FBS (vol/vol), 50U/mL penicillin and 0.1mg/mL streptomycin at 37C in 5% CO_2. WI38 was grown in DMEM supplemented with 15% FBS (vol/vol), 50U/mL penicillin and 0.1mg/mL streptomycin, 0.1 mM NEAA at 37C in 5% CO_2.

Establishment of USP36 Inducible Cell Line. To generate tet-inducible expression of USP36, we transfected T-Rex–U2OS cells that are hygromycin resistant (Invitrogen) with pcDNA4-TO–USP36, which contained the Zeocin selection marker. Cells were then cultured in DMEM supplemented with 10% tetracycline system-approved FBS (Clontech) in selection medium (50 μg/mL of hygromycin and 100 μg/mL of Zeocin) for roughly two weeks. Single colonies were isolated and expanded. Individual clones were then screened by treating with doxycycline (2ug/mL) for 48 hours and assayed by western blot for Flag expression. Positive clones were frozen at an early passage.

Lentivirus. Generation of lentivirus was described in (Sun et al. 2015). Briefly, 293FT were transfected with VSVG, pLP1, and pLP2, and vectors containing shRNAs targeting USP36 (see below for sequence), using Calcium Chloride (Promega). 48 hours later, media was harvested that contained virus. For infections, cells were pretreated with polybrene (6 μg/mL) for at least 2 hours. Viruses were then added directly to the media and left for 72 hours, followed either by passage or harvested for immunoblot. Lentiviral vectors encoding shRNA against USP36 were purchased (Open Biosystems). The shRNA sequences are listed in Table 7

MTS assay to measure cell viability. To study cell proliferation following knockdown of USP36, the percent of viable cells was calculated using the colorimetric MTS assay (CellTiter 96 Aqueous assay, Promega), following manufacturers instructions. Tetrazolium in the MTS reagent is reduced to formazan dye in metabolically active cells. The formazan dye is quantified by measuring absorbance at 490 mm and this is proportional to the number of viable cells. Results were then plotted relative to the scramble control. Briefly, cells were infected with lentivirus containing either scramble shRNAs or shRNAs targeting USP36 for 72 hours in a 96 well plate (triplicate wells). Media was replaced prior to starting MTS assay. 20ul of MTS reagent was added to each well and the plate was incubated at 37^0C for 3 hours at 5% CO_2. Absorbance was then read at 490 nm. Results for each shRNA were then plotted relative to the scramble control.

IncuCyte proliferation assay. Cells were plated in 12 well plates so that their density reached approximately 15% 24 hours after plating. After allowing cells to attach and spread out for 24 hours, plates were then loaded onto the IncuCyte Zoom System (Essen Bioscience), which gives kinetic information about cells growing in culture by taking multiple images from each well at fixed intervals of time. The IncuCyte Zoom software calculates the % confluence for each image and this was plotted against time for each sample (triplicate wells).

Transfection. Cells were plated 24 hours prior to transfection such that they would be roughly 60% confluent on the day of transfection. Transfection was carried out using Mirus reagent and the manufacturer protocol. Briefly, plasmids (2-4ug / 60mM plate) were added to tubes with 200ul of DMEM (serum free). At the same time 2ul / ug of mirus was added to a master mix in DMEM. The mixtures sat for ~10 minutes at room temp. 200ul of Mirus mater mix was added to each tube containing plasmid in DMEM. The plasmid/reagent mixture was left at room temperature for 22 min. This was then added directly to cells drop by drop. Cells were harvested 48 hours post-transfection.

Immunofluorescence Staining. H1299 cells were transfected with HA-MDM2 and Flag-USP36 for 48 hours. They were then washed in ice-cold PBS 2X on the plate. 4% Paraformaldehyde was added to plates for 5 minutes to fix cells. Cells were then washed twice with ice cold PBS. 0.1% Triton-X was placed on plate

and gently rocked for 3 three-minute intervals in order to permeabilize cells. Again cells were washed twice in ice cold PBS. 8% BSA was added to the plate for 30 minutes to block. Cells were incubated with primary antibody for 45 minutes followed by 3 washes in PBS (5 minutes each). This was followed by incubation with secondary antibody (Alexa Fluor 488 and Alexa Fluor 546 Molecular Probes) and DAPI stain (4′,6′diamidino-2-phenylindole) for 45 minutes and 3 PBS washes. Cells were then visualized on the Evos microscope.

Edu Labeling. Adapted from (Qu et al. 2011). Following knockdown of USP36 for 72 hours, cells were incubated with 10 μM EdU for 6 h at 37C. Plates were then washed twice in PBS and fixed in 4% PFA for 8 min at room temp. PFA was removed and plates were again washed twice in PBS. This was followed by permeabilization using 0.5% Triton X-100 in PBS, incubated for 5 min at room temperature, followed by 2x PBS wash. 0.5mL of EDU staining buffer was added to each plate and incubated for 30 min. Cells were then washed twice with 1mL of 3% BSA in PBS. 1 mL of DAPI in 1% BSA in PBS (1ug/mL) was added to plates and incubated for 10 min followed by three PBS washes. The number of EDU labeled cells out of the total number of DAPI cells was counted using ImageJ software and graphed relative to the scramble control.

Senescence associated beta-galactosidase. As described in (Nature protocol), with some minor modifications (Debacq-Chainiaux et al. 2009). WI38 cells were infected with lentivirus containing shRNAs for 7 days. Cells were then washed

PBS, fixed for 5 min at room temperature 4% paraformaldehyde followed by PBS wash 2x. Fresh SA-β-Gal stain solution (40mM citric acid/ Na phosphate buffer (ph 6.0), 5mM $K_4[Fe(CN)_6]3H_2O$, 5mM $K_3[Fe(CN)_6]$, 150mM sodium chloride, 2mM Magnesium chloride, 1mg/ml^{-1} X-gal in distilled water) was added to plates and then incubated at 37°C with no C02 for 16 hours. Cells were washed 3 times with PBS and then either dried with methanol or permeabilized with 0.5% triton-X for 10 min. followed by addition of DAPI and 4 PBS washes. Flouromount was used to adhere cover slip. Percentages were calculated based on the number of β-gal+ cells out of total number of cells counted from random fields.

Protein Half-life assay. Cells were infected with indicated shRNAs or transfected with plasmids for 48 hours. Prior to harvest, plates were treated with cyclohexamide (50ug/mL), to inhibit new protein synthesis, for the times indicated. All cells were harvested at the same time and assayed by immunoblot for MDM2 and tubulin. Image J was used to quantify western bands. Once values relative to tubulin loading were determined, they were plotted, the Y-axis was the percentage of protein present relative to time 0 (100%) and the X-axis was time. Linear trend lines were determined for each sample. This allowed us to calculate the time in which 50% of the protein was left.

Immunoblot. Cells were lysed in NP40 lysis buffer supplemented with protease inhibitors for one hour on ice. Lysates were cleared by centrifugation. Equal amounts of protein were run in an SDS-PAGE gel. Proteins were transferred onto

nitrocellulose membranes using TransBlot (15mV for 45 minutes). Membranes were blocked in 5% Milk-TBST for 1 hour. Primary antibodies (in 1X TBST) were incubated for 45 minutes, followed by 4 washes (8 minutes each). Secondary antibodies were also incubated for 45 minutes, followed by 4 washes. Finally proteins were detected with the ECL-Western blotting system (Amersham Biosciences).

Co-immunoprecipitation. Cells were lysed in NP40 lysis buffer for one hour on ice, followed by centrifugation to clear the lysate. Supernatants were collected and a small portion was set aside for immunoblot analysis (Inputs). For Co-IP assays, 20ul of either protein A or protein G were added to lysates and tumbled at 4C overnight. The next morning, IP antibody was added for 3-4 hours, and beads were then washed four times (8 minutes each) with NP40 lysis buffer. Bound proteins, alongside inputs were detected by immunoblot using the antibodies indicated in figures.

Assaying Polyubiquitination in cells. H1299 cells were transfected with His-Ubiquitin, along with other indicated plasmids for 48 hours. Prior to harvesting, cells were treated for six hours with 20 μM of MG132. Cells were harvested by scraping in ice cold PBS. They were then split into two aliquots, one for immunoblotting. The other aliquot was lysed in Buffer I, followed by incubation with 50ul of Ni-NTA beads (QIAGEN) at room temperature for 4 hours. Beads were then washed one time in Buffer I, one time in Buffer II, one time in Buffer III

followed by elution in Buffer IV. (200 mM imidazole, 0.15 M Tris-HCl [pH 6.7], 30% glycerol, 0.72 M β-mercaptoethanol, and 5% SDS). Eluted proteins were then analyzed by immunoblot with indicated antibodies.

Cell Fractionation Assay. 2 10-cm plates of H1299 were harvested at confluence by trypsinization. Cells were spun down at 1000 rpm for 4 min and washed once in PBS. Pellets were resuspended in 500ul of ice-cold Buffer A and kept on ice for 5 min. To release nuclei cells were dounced 10-12 times on ice with a pestle. Homogenate was transferred to an eppendorf tube and spun down at 4C for 5 minutes (228g). The supernatant was discarded and the pellet was resuspended in 300 ul of ice-cold S1. This was gently placed on top of a sucrose cushion (300ul of ice-cold S2). This was then spun down at 4c for 5 minutes (1430g). The supernatant was discarded. Pellet was then resuspended in 300ul of S2 and sonicated 5 times 10 sec, half power with one minute on ice between sonications. This was layered over 300ul of cold buffer S3, followed by centrifugation for 10 minutes at 4C (2800g). The supernatant was saved at the nucleoplasmic fraction (vol: 600ul). The pellet containing the nucleolar fraction was then washed with 0.5 ml of S2 and centrifuged for 5 minutes at 2800g. Finally it was resuspended in the same volume as the nuclear fraction (600ul of RIPA). Equal volumes were then compared by immunoblot.

MNase digestion for chromatin fractionation. As previously described with modifications, cells were trypsinized and washed 2x in cold PBS. Pellets were

then resuspended in buffer AA and incubated on ice for 5 minutes. Cytosolic proteins were separated from nuclei by centrifugation (4 min, 1,300 × g) and then discarded (cyto). The remaining nuclear pellet (A) was washed in buffer AAA and split into 2 tubes (A and B). One tube (A) was treated with MNase to release chromatin-bound proteins the other was not (B). Tube (A) was resuspended in buffer AAA and 1X MNase buffer, 1% BSA and 1ul MNase. Reactions were incubated for 5 min at 37C followed by 20 minutes at room temp. The reaction was stopped by adding 1mM EGTA. Digested nuclei were spun down at 1,300 × g for 4 min. Nuclei from both digested and undigested samples were then lysed in solution B for 30 min on ice, followed by centrifugation (4 min, 1,700 × g). The supernatant (A-SN, B-SN), contained soluble chromatin fragments separated from insoluble chromatin (A-P, B-P). Equal volumes were assayed by western blot. Scheme shown in Appendix 4.

Histone extraction. Cells were harvested by trypsinization followed by a wash with PBS. Pellets were resuspended in TEB buffer so that the cell density was roughly 10^7 / mL for 10 minutes on ice. Lysates were then centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded. Cells were washed once in TEB, then centrifuged at 2000 rpm for 10 minutes. Pellets were resuspended in 4X the original volume of 0.2N HCl and placed at 4C overnight. The next morning, they were centrifuged at 2000 rpm for 10 minutes. The supernatant was harvest and then neutralized by adding an equal volume of 0.2N NaOH.

In vitro H2bub2 deubiquitination assay. Extracted histones were incubated with bacterially purified His-USP36 WT or His-USP36 CA at room temperature overnight. The entire reaction was then assayed by immunoblot.

GST pulldown assay. His-tagged USP36 (1-800), USP36 C131A (1-800) and MDM2 proteins were expressed in *E. coli* and purified by Ni-NTA pulldown. GST fusion proteins were also purified from *E. coli* using glutathione-Sepharose 4B beads (Sigma). 200ng of GST-tagged proteins still bound to beads was incubated with 200ng of His-tagged proteins. The mixtures were 1X in BC100 + 0.1% Nonidet P-40, twice in SNNTE and once in RIPA. Bound proteins were run on SDS-PAGE gels, followed by immunoblot analysis.

RT-qPCR. Total RNA was isolated from cells using Trizol reagent as per the manufacturer protocol (Invitrogen). RNA was quantified and 500ng was used in for cDNA synthesis using the iScript reagent kit (Biorad). Following cDNA synthesis, cDNA was diluted 1:3 and 2 ul was used in subsequent qPCR reactions. qPCR was carried out using SYBR green Mix (Biorad) with a final volume of 20ul per well. qPCR was performed on an ABI StepOne^{TM} real-time PCR system (Applied Biosystems)

Chromatin Immunoprecipitation. Cells were fixed by adding formaldehyde solution directly to the medium (1% final concentration), for 10 minutes at room temperature. After fixation, glycine was added to a final concentration of 0.125M

for 5 minutes at room temperature. Cells were then washed twice in PBS. To extract chromatin, cells were resuspended in RIPA lysis buffer. Lysates were then sonicated to yield chromatin fragments of approximates 600-1000bp. The sonication conditions were 5 cycles for 12 sec, each at max power and placed on ice for 2 minutes between cycles. Lysates were then spun down at 130,000 rpm for 15 minutes. Extracts were cleared for 30 min at 4C with 50% slurry protein A. 2ug of the appropriate antibody were added and the tubes were tumbled overnight at 4C. The next morning, 50ul of protein A were added to each tube and tumbled for a subsequent 2 hours. Immunoprecipitates were washed six times for 15 min each with ChIP buffer, followed by two TE washes, the first for 30 min, the second for 5 minutes. To elute fragments, 250ul of elution buffer was added to each tube and rotated for 15 min at room temperature. 200ul of elution buffer was also added to 50ul of each input. Samples were then spun down and the supernatant was transferred to a new tube. To reverse crosslinking, 12ul of 5M NaCl was added to each sample and incubated overnight at 65C. The next morning, all samples were cleaned up using the Qiagen PCR cleanup kit. 2ul were then used for qPCR.

TCGA analysis of USP36 expression in cancer. Using the cBio portal, data was compiled for all cancer types available as of May 2013. Putative copy-number alterations from GISTIC, mutations, and mRNA expression data were examined for each data set. The number of patients with these alterations was

divided by the total number of patients with available data and this was graphed as percentage altered. http://www.cbioportal.org

5 Summary and Discussion

This body of work extends our knowledge about the deubiquitinating enzyme USP36. First we show that USP36 conserves the function of its yeast and drosophila orthologs as a transcriptional repressor capable of deubiquitinating H2bub1. Overexpression of USP36 dramatically reduces global H2bub1 levels. This is dependent on its deubiquitinating enzyme activity and most likely occurs on chromatin. The mechanism through which USP36 is recruited to chromatin appears to be an interaction with histone 2b, as we have found that it interacts with H2b both in *vitro* and within cells. Whether this interaction is direct or mediated indirectly through other partner proteins such as RNF20 is an open question. Overall, our results strongly indicate that USP36 may regulate gene expression by modulating H2bub1 levels.

Using the p21 gene locus as a model, we have demonstrated that USP36 negatively regulates gene expression through deubiquitination of histone 2b. We show that USP36 binds to the p21 gene, primarily within the transcribed region. Knockdown of USP36 significantly increased H2bub1 within p21, along with a corresponding increase in H3K4me3 near the transcription start site. Thus, we conclude that as a conserved H2bub1 deubiquitinase, USP36 negatively regulates p21 expression. Interestingly, there may be other cooperating mechanisms in place that reinforce p21 repression. In particular, we have previously shown that USP36 can deubiquitinate and stabilize c-Myc, which is known to repress p21 transcription. While, USP36 regulates c-Myc within the

nucleolus, we speculate that deubiquitinated c-Myc is then translocated back to the nucleoplasm, where it may potentially repress p21. Therefore, it will be interesting to test whether USP36 utilizes multiple mechanisms to suppress the cell cycle inhibitor p21 (Figure 5.1).

In addition to specifically regulating p21 expression, USP36 mediated deubiquitination of H2bub1 may have broader consequences. For example, studies in breast and gastric cancer have shown that global H2bub1 levels decrease with tumor progression. Interestingly, decreasing H2bub1 through depletion of RNF20 results in increased expression of tumor suppressor genes as well as a reduction in oncogenes (Shema et al. 2008). Consistently, the enzymes regulating H2bub1 are deregulated in cancer. For example, RNF20, the E3 ligase mediating H2bub1 has been implicated in tumor suppression. Functionally, depletion of RNF20 was shown to increase tumor burden in mouse xenograft model (Shema et al. 2008). Furthermore, in breast cancer RNF20 expression is silenced by promoter methylation (Prenzel et al. 2011). In contrast, USP22, the primary H2bub1 DUB is overexpressed in a variety of cancer types (Glinsky 2005; Li et al. 2013; Y.-L. Liu et al. 2011; Lv et al. 2011; Piao et al. 2012; Piao et al. 2013; H. Wang et al. 2013; Yang et al. 2013; Y. Zhang et al. 2011; Yang et al. 2011). Additionally, several studies have shown a correlation between decreased H2bub1 levels and elevated USP22. Interestingly, we have observed that USP36 may also antagonize RNF20-mediated H2b monoubiquitination as well. Through examination of global H2bub1 by immunoblot, we have observed that USP36 partially reverses RNF20 ubiquitination of H2b (Appendix 2). Future

correlational studies that examine USP36, USP22, RNF20 and H2bub1 at the protein level and preferably within the context of human tumors, will provide a more complete picture as to how these proteins cooperate to regulate H2bub1

While the first half of this thesis focused on the ability of USP36 to regulate mono-ubiquitination of a target, in the second half, we describe a novel role for USP36 in regulating the stability of both p53 MDM2 through removal of poly-ubiquitin chains. p53 is an essential tumor suppressor that coordinates a cell's response to a variety of insults. Under normal conditions p53 is maintained at low levels by the E3 ligase, MDM2, which mediates p53 degradation through the ubiquitin-proteasome system. Additionally, MDM2 regulates p53 activity through ubiquitin independent mechanisms. For example, MDM2 interacts with p53's N-terminal transactivation domain and blocks the recruitment of co-activators to target genes. In response to stress, the MDM2-p53 complex is disrupted and this results in increased stabilization and activity of p53. Accumulating studies have established that the p53-MDM2 axis links cellular stress to cell growth and ribosome biogenesis through the nucleolus. However, whether a nucleolar DUB can reverse the ubiquitination of p53 and/or MDM2 was lacking.

Here, we show that USP36 interacts with, deubiquitinates, and stabilizes both MDM2 and p53. USP7 is the only other DUB also known to positively regulate both MDM2 and p53. USP7 interacts directly interact with both MDM2 and p53 through the same binding domain. Its binding affinity is much greater towards MDM2 than p53 as shown by competition assay. Therefore, under

normal conditions, MDM2 is the preferred target of USP7. Upon DNA damage, it preferentially binds to and stabilizes p53. The differential contextual regulation of p53/MDM2 by USP7 explains how and why this DUB targets both p53 and MDM2. As USP36 appears to have to similar positive activity towards both proteins, future work should aim to identify the context in which USP36 may prefer functional regulation of one target over the other (i.e. p53 versus MDM2).

One unexplained observation that we have made is that, in spite of robust increases in p53 levels upon USP36 overexpression, canonical p53 target genes are not induced (data not shown). There are two main competing hypotheses that could account for this effect. First, USP36 may stabilize p53; yet interfere with its ability to transactivate target genes. There are a number of possible mechanisms and hypotheses, explored below, which could help to explain how this might occur (if it is occurring). Second, USP36 mediated stabilization of p53, may positively regulate a set of untested p53 target genes. Although, we haven't seen induction of canonical targets, it may be that USP36 regulates a very specific p53-controlled program. Our understanding of the transcriptional network regulated by p53 has grown immensely in recent years. From these studies, a list of 129 high confidence targets has been curated. This list includes tissue specific targets of p53, as well as a number of targets that fall outside of the canonical pathways regulated by p53 (i.e. apoptosis, cell cycle control, senescence) (Riley et al. 2008). A complete and targeted examination of how USP36 regulates this entire network would be a first step at determining whether and if USP36 positively regulates p53 activity. Future experiments may include

either global RNA sequencing analysis, or determining expression of a smaller custom panel of high confidence p53 targets upon both knockdown and overexpression of USP36. Additionally, it may be necessary to examine expression levels of p53 targets under stress conditions, particularly due to the fact that proteins levels alone do not necessarily correlate with activity. Therefore, functional assays and modulation of downstream targets must be the endpoint in determining how USP36 regulates this pathway, as opposed to looking at protein stability alone.

As mentioned, an alternative hypothesis is that USP36 stabilizes p53 but interferes with its ability to transactivate target genes. If the above analysis did not yield any significant changes in target gene expression or if USP36 instead appeared to preferentially repress p53 activity, there are a number of scenarios that could be explored to determine the mechanism of repression. Initially, p53 ChIP analysis should be carried out at a number of loci in cell lines expressing wild-type functional p53 with USP36 overexpression or knockdown. If USP36 reduces p53 promoter binding, a next step would be to determine if it interferes with tetramerization of p53, which is known to be essential for transcriptional activity, or whether it effects post-translational modifications required for promoter binding. Crosslinking agents could be used to analyze p53 tetramers by western blot upon USP36 overexpression or knockdown.

If ChIP analysis reveals that p53 promoter binding is not disrupted by USP36, yet we still fail to observe transcriptional activation, we could test whether USP36 stabilizes the interaction of MDM2 with p53 at the target

promoters through ChIP analysis of MDM2. MDM2 is known to inhibit p53 transactivation by binding to its transactivation domain and blocking the recruitment of co-activators. The stable presence of the MDM2/MDMX complex at p53 target genes was shown to be repressive (Oliner et al. 1992; Thut, Goodrich, and Tjian 1997). Interestingly, acetylation of p53, releases it from interacting with MDM2 and MDMX (Tang et al. 2008). This allows for rapid activation of target genes. Mutating p53 so that its incapable of being acetylated causes it to retain a strong interaction with MDM2 and MDMX enforcing the repressed state (Tang et al. 2008). MDM2 can also repress p53 activity through a repression domain (50-220 amino acids and partially encompassing the central acidic domain) that can interact with TFIIE, a component of the basal transcription machinery involved in regulating RNA polymerase II elongation (Thut et al. 1997). TFIIE then recruits TFIIH, which functions as both a helicase and an ATPase. Although not entirely worked out, it is hypothesized that MDM2's interaction with TFIIE, blocks its ability to recruit TFIIH (Thut et al. 1997). Thus, if USP36 stabilizes the p53-MDM2 complex at p53 target gene promoters, we would expect reduced p53 acetylation at the promoters. We could perform ChIP using anti-acetylated p53 (e.g. acetyl-K382) to address this question.

Together, our work has elucidated two novel functions of USP36 in controlling cell growth and proliferation, in addition to our recent finding showing that USP36 deubiquitinates and stabilizes c-Myc (Sun et al. 2015). Whether these pathways participate in crosstalk with each other is an important question for future exploration. The current data clearly suggest that USP36 is essential

for cell growth and proliferation, owing to its key roles in ribosomal biogenesis in the nucleolus and regulating H2bub1 and gene expression (e.g. suppressing p21 expression) in the nucleoplasm (Figure 5.1). In any case, our observations suggest that USP36 may link cell growth processes, specifically ribosome biogenesis, to cell division. Detailed analysis of USP36 functions throughout the cell cycle will should be a critical component to future studies.

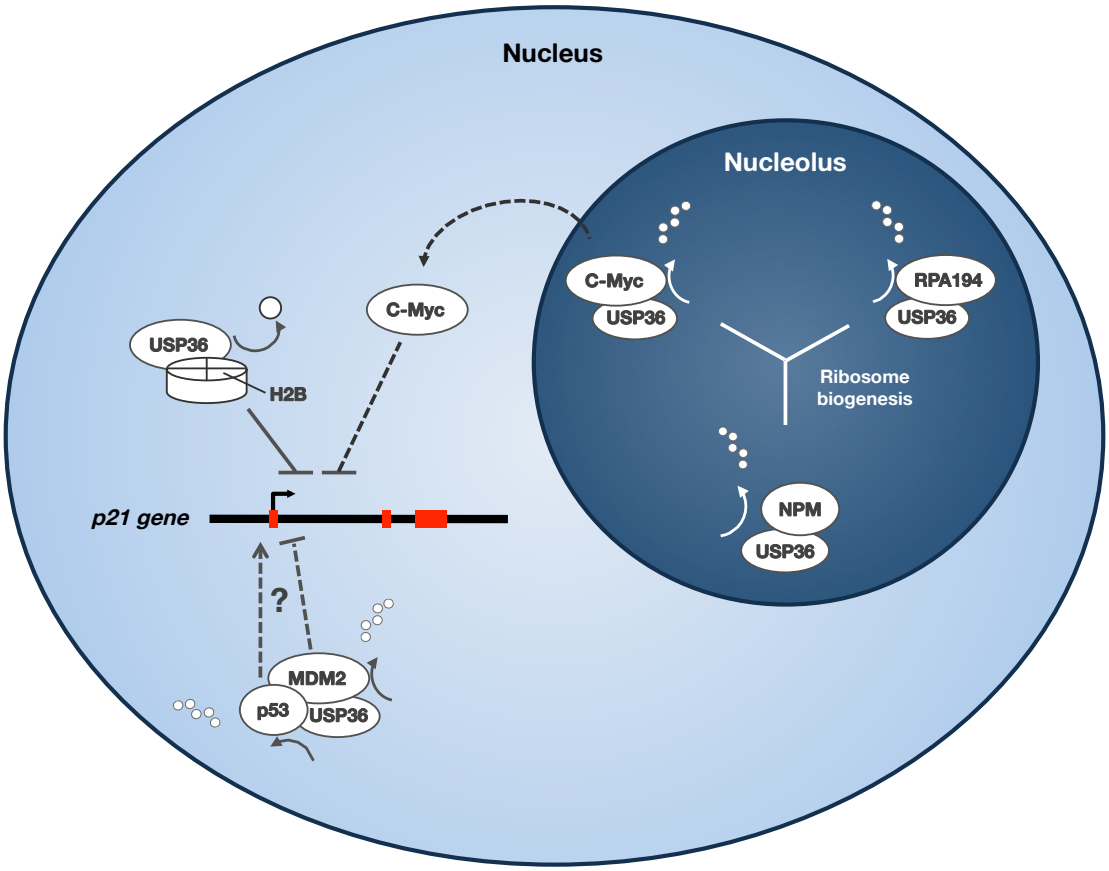
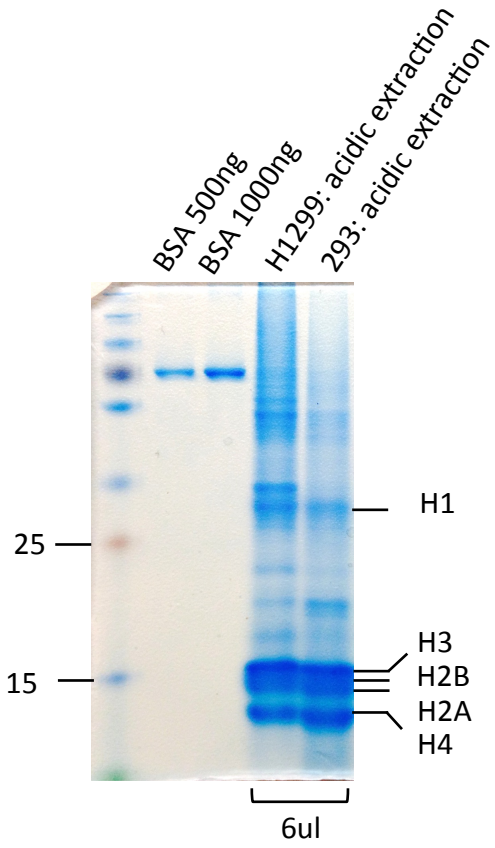


Figure 5-1: USP36 functions within the nucleus and the nucleolus.

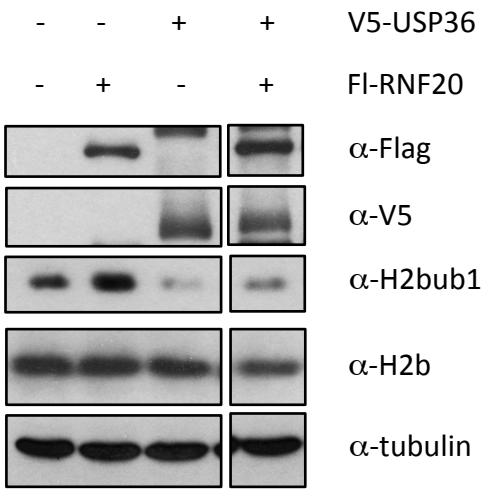
UPS36 is primarily localized in the nucleolus where it plays a critical role in promoting ribosome biogenesis by deubiquitinating and stabilizing NPM, RPA194, and c-Myc. In this thesis, I identified additional functions of USP36 in the nucleoplasm: deubiquitinating H2bub1 and the p53-MDM2 pathway. I show that USP36 deubiquitinates H2bub1 in the p21 gene body and suppresses p21 expression independently of p53. c-Myc stabilized by USP36 in the nucleolus may translocate into the nucleoplasm where it can suppress p21 as well. I also show that USP36 is a novel deubiquitinase of both p53 and MDM2. Whether the USP36 mediated stabilization of p53 and MDM2 by contributes to the regulation of p21 expression remains to be explored. Dotted lines indicate unknowns.

6 Appendices



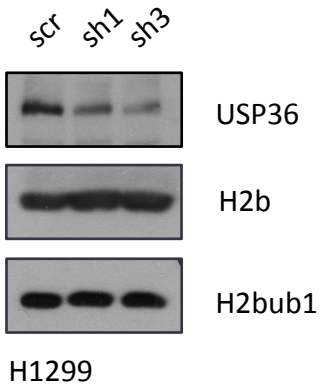
Appendix 1: Coomasie Stain of Purified Histones.

Histones were extracted from H1299 and 293 cells and run on an SDS-PAGE gel, followed by staining with coomasie to visualize. Histone position is noted in the figure.



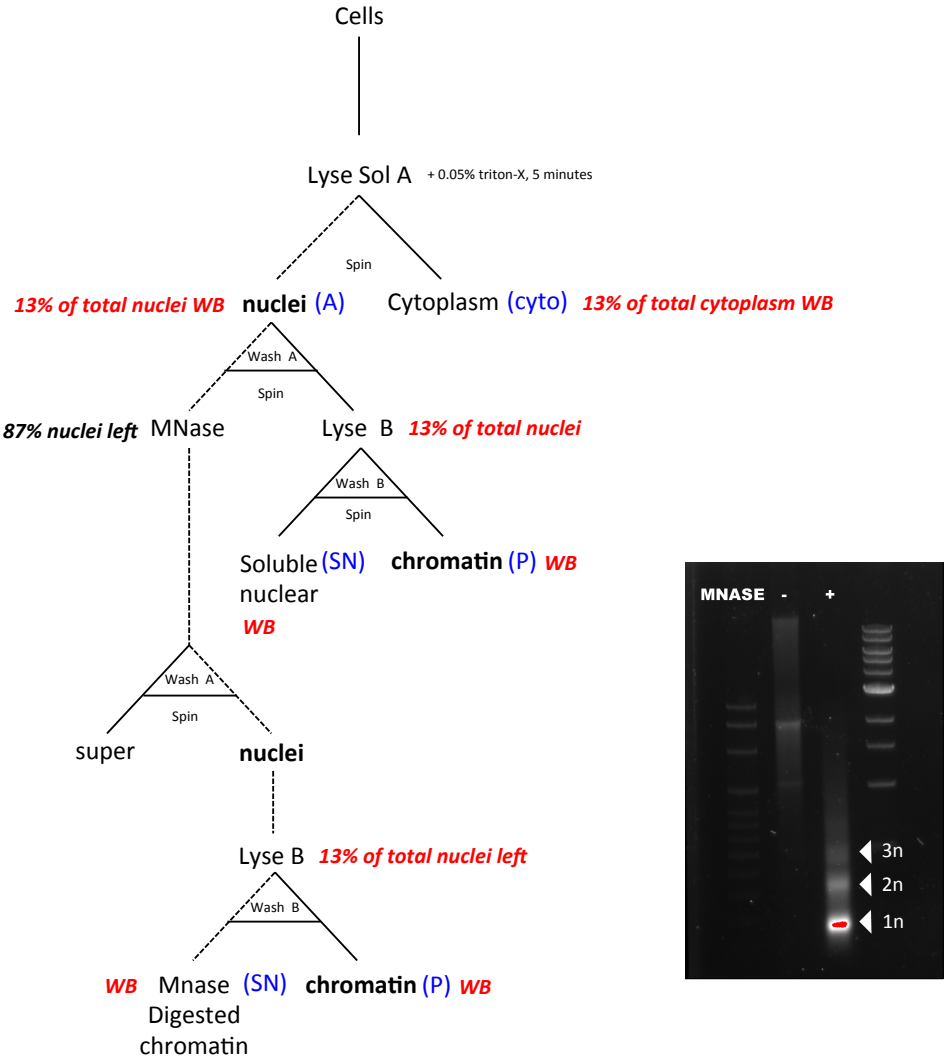
Appendix 2: USP36 partially reverses RNF20 mediated H2bub1.

H1299 cells were transfected with either V5-USP36 and or Flag-RNf20 and assayed for H2bub1.



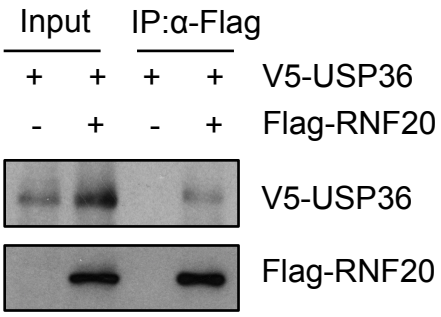
Appendix 3: Depletion of USP36 does not effect global H2bub1 levels.

H1299 cells were infected with sh-RNA targeting USP36 or a scramble control, followed by immunoblot assay for indicated antibodies.



Appendix 4: Scheme for Mnase fractionation assay.

Visual representation of MNAse fractionation, a detailed description can be found in methods. DNA with and without MNAse was run on an agarose gel to show that the digestion was complete. The labels correspond to DNA wrapped in nucleosomes (1n = one nucleosome). The majority of nucleosomes are single, indicating adequate digestion.



Appenix 5: USP36 interacts with RNF20

H1299 cells were co-transfected with V5-USP36 and Flag-RNF20. Lysates were then analyzed by immunoblot for the antibodies shown.

Acknowledgements for contribution of work.

Figure 3-1A: Co-immunoprecipitation of HA-MDM2 and Flag-USP36 was conducted by Xiao-Xin Sun

Figure 3-4A: USP36 stabilizes MDM2 in a dose dependent manner was contributed by Xiao-Xin Sun

Figure 3-4B: USP36 stabilizes MDM2 but USP36^{C131A} does not was contributed by Xiao-Xin

Figure 3-9A: USP36 deubiquitinates p53 was contributed by Yuegang Wang

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