THE MULTIFUNCTIONAL PROTEIN DAXX: STUDIES OF ITS BIOLOGY AND REGULATION,
AND DISCOVERY OF A NOVEL FUNCTION

Trisha Agrawal

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Supervisor of Dissertation

_____

Xiaolu Yang, PhD, Professor of Cancer Biology

Graduate Group Chairperson

_____

Julie Blendy, PhD, Professor of Pharmacology

Dissertation Committee

Richard K. Assoian, PhD, Professor of Pharmacology

J. Alan Diehl, PhD, Professor of Cancer Biology

Donna L. George, PhD, Associate Professor of Genetics

James Shorter, PhD, Associate Professor of Biochemistry and Biophysics

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DEDICATION

To my parents, without whose love, support and positive attitude, I could not have achieved what I have achieved today.

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ABSTRACT

THE MULTIFUNCTIONAL PROTEIN DAXX: STUDIES OF ITS BIOLOGY AND REGULATION,
AND DISCOVERY OF A NOVEL FUNCTION

Trisha Agrawal

Xiaolu Yang, PhD

Daxx, a multifunctional protein with a diverse set of proposed functions, is ubiquitously expressed and highly conserved through evolution. A primarily nuclear protein, Daxx is able to regulate apoptosis, transcription, and cellular proliferation. Despite many studies into the function of Daxx, its precise role in the cell remains enigmatic. Herein, evidence is presented to expand upon the known anti-apoptotic function of Daxx, to establish Daxx as a novel molecular chaperone, and to further its repertoire of transcriptional targets. As an apoptotic inhibitor, Daxx is known to regulate p53 by stabilizing its main negative regulator, Mdm2, via formation of a ternary complex between Daxx, Mdm2, and Hausp. The present study reveals that DNA damage-induced phosphorylation of Daxx is an important step in the disruption of the Daxx-Mdm2-Hausp complex, allowing for p53 activation. A novel activity for Daxx is presented whereby it is able to modulate protein folding *in vitro* and *in vivo*. Daxx can refold and reactivate denatured substrates using its paired amphipathic helical and acid-rich domains. This finding was extended to p53, where Daxx was able to solubilize misfolded p53 both *in vitro* and *in vivo*. Further, this finding may provide a biochemical rationale as to the varied functionality of Daxx in the cell. Finally, a novel transcriptional target, Cdk6, is described for Daxx. Microarray analysis indicated that Cdk6 is a strongly downregulated gene upon Daxx silencing. Depletion of Daxx in various cancer and primary cell lines led to a decrease in Cdk6 protein levels. Additionally, Daxx can affect transcription of *Cdk6,*

and chromatin immunoprecipitation reveals that Daxx binds to the *Cdk6* promoter. Together, these results indicate that Daxx has potential functional plasticity and is involved in an array of cellular functions; in fact, cellular homeostasis relies on the proper execution of multiple biological processes. Elucidation of the biology of Daxx would not only provide insight into the regulation of these processes, but may also establish Daxx as a relevant therapeutic target.

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CHAPTER 1: INTRODUCTION

Daxx

The multifunctional protein Daxx (death domain-associated protein) has been implicated in apoptosis, transcription, and cellular proliferation. Despite extensive study into its function, its precise role in the cell is unclear and encompasses a seemingly divergent set of proposed activities. This dissertation aims to further understand the biological role of Daxx and the following section will give an overview regarding the structure, subcellular localization, and known functions of Daxx.

Discovery and Structure

Daxx was first identified by a yeast two-hybrid screen as an adaptor protein for the intracellular portion of the death receptor, Fas (CD95/Apo-1), and potentiates Fas-induced apoptosis via the c-Jun N-terminal kinase (JNK) pathway (Yang, Khosravi-Far, et al, 1997). Human Daxx is a protein of 740 amino acids and has no significant homology to known proteins. It is ubiquitously expressed with especially high expression in the testes (Kiriakidou, Driscoll, et al, 1997; Yang, Khosravi-Far, et al, 1997). Daxx is poorly characterized at the structural level; however, among known domains it contains two N-terminal paired amphipathic helical domains, a coiled-coil domain, an acid-rich (D/E) domain, and a C-terminal Serine/Proline/Threonine-rich domain (Figure 1.1). Daxx also contains two SUMO-interacting motifs (SIMs), one each within its N- and C-terminus, which are important for its subcellular localization and association with other proteins (Escobar-Cabrera, Okon, et al, 2011; Lin, Huang, et al, 2006; Ryu, Chae, and Kim, 2000; Santiago, Godsey, et al, 2009).
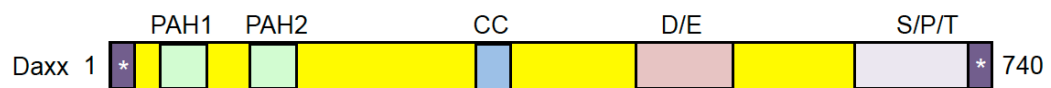
Figure 1.1. Schematic depicting structural domains within Daxx.
PAH – paired amphipathic helices; CC – coiled coil domain; D/E – acid-rich region;
S/P/T – serine/proline/threonine-rich region. Asterisks indicate SUMO-interacting motifs.

Cellular Localization

   Subsequent to its initial discovery as a cytoplasmic protein, many studies emerged confirming the cytoplasmic localization of Daxx and validating its pro-apoptotic function (Chang, Nishitoh, et al, 1998; Gongora, Stephan, et al, 2001; Ko, Kang, et al, 2001; Lalioti, Vergarajauregui, et al, 2002; Perlman, Schiemann, et al, 2001). In the cytoplasm, Daxx associates with the apoptosis signaling kinase 1 (ASK1) and this association can be inhibited by heat shock protein 27 (Hsp27) (Chang, Nishitoh, et al, 1998; Charette, Lavoie, et al, 2000; Ko, Kang, et al, 2001; Song, and Lee, 2003). Despite evidence of Daxx being a pro-apoptotic protein residing mainly in the cytoplasm, it later became clear that Daxx is predominantly a nuclear protein, which may be translocated to the cytoplasm in response to certain stimuli; for example, FAS stimulation and glucose deprivation (Lindsay, Giovinazzi, and Ishov, 2009; Song, and Lee, 2004; Yang, Khosravi-Far, et al, 1997).

   In the nucleus, Daxx localizes in promyelocytic leukemia-nuclear bodies (PML-NBs), macromolecular complexes that regulate a variety of cellular processes including tumor suppression and transcriptional repression (Bernardi, and Pandolfi, 2007; Li, Leo, et al, 2000; Torii, Egan, et al, 1999; Zhong, Salomoni, et al, 2000). An essential component of PML-NBs is the PML protein, which when SUMOylated, is recognized by the C-terminal SIM on Daxx and leads to the accumulation of Daxx at PML-NBs (Ishov, Vladimirova, and Maul, 2004; Lin, Huang, et al, 2006). Daxx displays aberrant localization in acute promyelocytic leukemia (APL) cells, where the PML-NB structure is disrupted (Zhong, Salomoni, et al, 2000). Daxx recruitment to PML-NBs is important for many of its functions including apoptotis and transcription (Li, Leo, et al, 2000; Torii,

Egan, et al, 1999). For example, in *PML* -/- B-lymphocytes, apoptosis is impaired due to the delocalization of Daxx from the PML-NBs (Zhong, Salomoni, et al, 2000).

In addition to its accumulation at PML-NBs, Daxx is localized to heterochromatin in a cell cycle-dependent manner (Hollenbach, McPherson, et al, 2002; Ishov, Sotnikov, et al, 1999; Ishov, Vladimirova, and Maul, 2004). This localization occurs via the association of Daxx with the α-thalassemia/mental retardation syndrome protein (ATRX), a putative member of the SNF2 family of ATP-dependent chromatin remodeling proteins (Tang, Wu, et al, 2004; Xue, Gibbons, et al, 2003). The N-terminus of Daxx interacts with ATRX and this association depends upon the phosphorylation of ATRX (Ishov, Vladimirova, and Maul, 2004). Ishov, et al found that Daxx and ATRX associate at PML-NBs during most of interphase. Daxx then leaves the PML-NBs and is actively recruited to heterochromatin during late S phase via association with phosphorylated ATRX and loss of Daxx leads to prolongation of S phase of the cell cycle (Ishov, Vladimirova, and Maul, 2004). Until recently, there was no explanation as to why Daxx would associate with heterochromatin during such a short window of time during the cell cycle. The newly discovered function of Daxx as a histone chaperone may shed light on this phenomenon and will be discussed in a later section.

Function

Apoptosis

Daxx initially appeared to be a pro-apoptotic protein; however, several lines of evidence indicate that Daxx may also have an anti-apoptotic function. Disruption of the Daxx gene in mice leads to embryonic lethality before E8.5 (Michaelson, Bader, et al, 1999). *Daxx* -/- embryonic stem (ES) cells show higher rates of spontaneous apoptosis,

suggesting that Daxx has an anti-apoptotic role that is important during development. Further, Daxx silencing in various cell lines sensitizes cells to apoptotic stimuli, verifying Daxx as an important anti-apoptotic modulator (Chen, and Chen, 2003; Michaelson, and Leder, 2003).

Additionally, research from our lab shows that Daxx has a critical role in regulating the tumor suppressor p53 by stabilizing mouse double minute 2 (Mdm2), the major p53 negative regulator (Tang, Qu, et al, 2006). Daxx forms a complex with Mdm2 and herpesvirus-associated ubiquitin-specific protease (Hausp) that serves to stabilize Mdm2, leading to the ubiquitination and degradation of p53 (Figure 1.2). Upon DNA damage, the Daxx-Mdm2-Hausp complex dissociates, resulting in Mdm2 degradation and allowing for the activation of p53-mediated apoptosis. The disruption of this complex is mediated by the protein kinase ATM (ataxia telangiectasia mutated); how ATM mediates the dissociation of the complex is the focus of chapter two.

Transcriptional Regulation

Due to the localization of Daxx to the nucleus, and in particular heterochromatin, several groups have focused their efforts on uncovering a possible role for Daxx in transcriptional regulation. Daxx has a diverse array of binding partners and many of these include transcription factors. Generally, Daxx is a transcriptional repressor as shown by its ability to repress the activity of several transcription factors including Pax3, ETS1, p53 and p73, NF-κB, CREB, glucocorticoid receptor, and HDAC2, among others (Gostissa, Morelli, et al, 2004; Huang, Chang, et al, 2012; Kim, Park, and Um, 2003; Lehembre, Muller, et al, 2001; Li, Leo, et al, 2000; Morozov, Massoll, et al, 2008; Park, Lee, et al, 2007). The transcriptional repressive activities of Daxx have been linked to its

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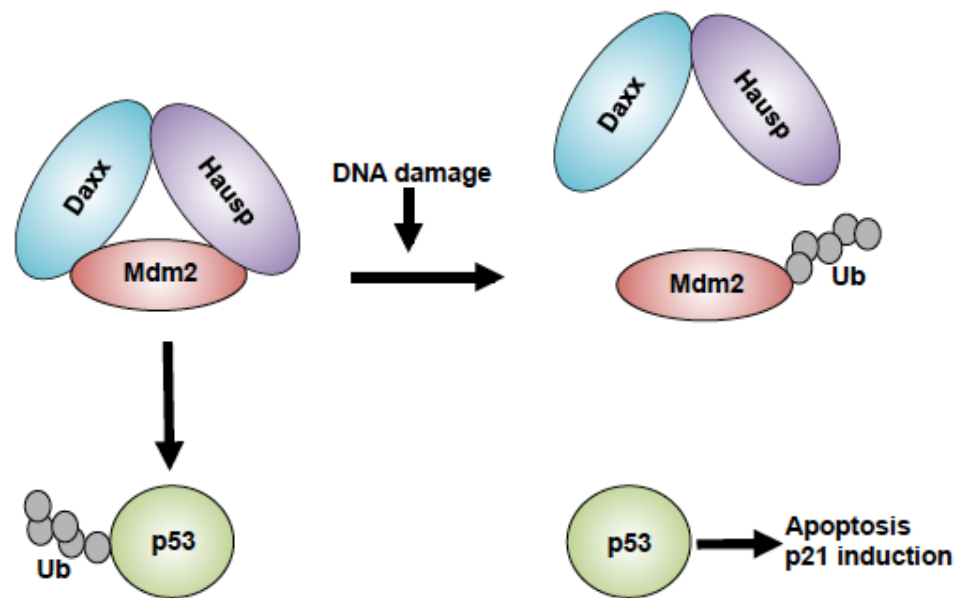
Figure 1.2. Illustration depicting role of Daxx in Mdm2 and p53 stability.
In unstressed cells, Daxx bridges the interaction between Hausp and Mdm2, preventing
Mdm2 ubiquitination. Upon induction of DNA damage, the complex dissociates leading
to Mdm2 degradation and p53-mediated apoptosis and p21 transcription.

association with ATRX and its delocalization from PML-NBs (Newhart, Rafalska-Metcalf, et al, 2012). PML can inhibit Daxx-mediated transcriptional repression via direct binding to Daxx and by subsequent recruitment of Daxx to PML-NBs. In *PML* -/- cells, Daxx forms aggregates around condensed chromatin, where it may exert its repressive activities (Zhong, Salomoni, et al, 2000). Daxx can also activate transcription of a few transcription factors, for example, Pax5 and HSF1, albeit under very specific cellular conditions (Boellmann, Guettouche, et al, 2004; Emelyanov, Kovac, et al, 2002).

Related to its activity as a transcriptional regulator, recent studies have implicated Daxx as a histone chaperone for the histone 3 (H3) variant, H3.3 (Drane, Ouararhni, et al, 2010; Elsaesser, and Allis, 2010; Lewis, Elsaesser, et al, 2010). Unlike the other H3 variants, H3.1 and H3.2, H3.3 is expressed throughout the cell cycle and is incorporated in chromatin in a replication-independent manner (Elsaesser, Goldberg, and Allis, 2010; Wu, Tsai, and Bonner, 1982). H3.3 is believed to be an important carrier of genetic information and while it was initially found solely at euchromatin, it has been shown to associate with regulatory elements and constitutive chromatin at telomeres (Lewis, Elsaesser, et al, 2010; Salomoni, 2013). Prior to the discovery of Daxx as a histone chaperone, the interaction between ATRX and H3.3 was considered to be essential for the maintenance of telomere structural integrity (Wong, McGhie, et al, 2010). Subsequent to this, two groups independently found that Daxx also associates specifically with H3.3 via the central domain of Daxx (Drane, Ouararhni, et al, 2010; Goldberg, Banaszynski, et al, 2010; Lewis, Elsaesser, et al, 2010). In accordance with its subcellular localization, Daxx was found in both cytoplasmic and nuclear H3.3 complexes. While Daxx alone was able to assemble chromatin *in vitro*, the deposition of H3.3 onto telomeres was dependent upon the formation of the Daxx-ATRX complex.

This Daxx-ATRX-dependent targeting of H3.3 to telomeres appears to suppress transcription over telomeric repeats in ES cells (Goldberg, Banaszynski, et al, 2010). Conversely, the deposition of H3.3 on pericentric heterochromatin by Daxx and ATRX leads to a greater abundance of mRNA transcripts at these regions (Drane, Ouararhni, et al, 2010). The role of H3.3 deposition is unclear, but the function of Daxx as a H3.3 chaperone provides a potential mechanism for its transcriptional regulatory activity. Indeed, a recent study shows that in neuronal cells, the deposition of H3.3 at a subset of immediate early genes (IEGs; Bdnf Exon IV, c-Fos, Egr2, and Dusp6) relies mainly on Daxx, leading to increased transcriptional induction of these genes (Michod, Bartesaghi, et al, 2012). It is clear that Daxx has the ability to regulate transcription but it is still nebulous if this regulation is inhibitory or activating. A novel transcriptional target of Daxx, Cdk6, will be explored in chapter four.

Cellular Proliferation and Cancer

Due to the interaction of Daxx with multiple proteins and the varying consequences of these interactions, it is plausible that Daxx has proliferative effects that may be mediated via its influence on apoptosis, transcription, p53 regulation, or histone chaperone activity. These effects may regulate the promotion of cancer and/or tumorigenesis. In fact, many groups have found that Daxx is involved in cellular proliferation and in turn, cancer progression. As previously discussed, Daxx can stabilize Mdm2 leading to the ubiquitination and degradation of p53. Depletion of Daxx in cells leads to greater apoptosis in an Mdm2- and p53-dependent manner (Tang, Qu, et al, 2006). This observation has led other groups to identify Daxx as an important modulator of p53 in cancer. Two studies from the same group indicate that Daxx is important for ovarian cancer progression (Pan, Yi, et al, 2013; Pan, Zhou, et al, 2013). They reported

that Daxx mRNA and protein levels were overexpressed in ovarian cancer cells lines and ectopic expression of Daxx led to increased ovarian cancer cell proliferation, migration, colony formation, and chemoresistance; depletion of Daxx had the converse effect. Additionally, transplantation of stable cells overexpressing Daxx into nude mice was capable of forming metastatic tumors. Daxx silencing led to enhanced senescence in mouse ovary surface epithelial (mOSE) cells in a p53/p21-dependent manner. In another study using pediatric acute lymphoblastic leukemia (ALL) cells, a pharmacological intervention was used to disrupt the Daxx-Mdm2-Hausp complex. The group found that Berberine, a potential herbal chemotherapeutic, was able to decrease Daxx transcription and protein levels, which led to reduced formation of the Daxx-Mdm2-Hausp complex. This allowed for p53 activation and greater cell death of the ALL cells (Zhang, Gu, et al, 2010).

The role of Daxx as a histone chaperone has also proven to be important for certain cancers, including pancreatic neuroendocrine tumors (PanNETs) and pediatric glioblastoma multiforme (GBM) (de Wilde, Edil, et al, 2012; Zhang, Francois, et al, 2013). When the exome of primary PanNETs was sequenced, somatic mutations in the genes encoding Daxx and ATRX were found in 42.6% of the PanNETs. There were mutually exclusive mutations in Daxx and ATRX, consistent with their presumptive cooperation in transcription and histone chaperone activity. Interestingly, patients with mutations in Daxx or ATRX had prolonged survival rates compared to patients without mutations in these genes (Jiao, Shi, et al, 2011). Due to the role of Daxx and ATRX in chromatin remodeling at telomeres, Heaphy, et al examined the status of telomeres in patients with PanNETs that contained mutations in Daxx or ATRX. A perfect correlation between loss of ATRX or Daxx function and the presence of alternative lengthening of

telomeres (ALT) was observed (Heaphy, de Wilde, et al, 2011). Further, mutations in

Daxx and ATRX have been found in cases of pediatric GBM. A recent study showed that

mutations in Daxx or ATRX were found in 31% of the tumor samples analyzed, and in

100% of samples that also harbored mutations in the gene that codes for H3.3, *H3F3A*

(Schwartzentruber, Korshunov, et al, 2012). Again, a mutation in Daxx or ATRX was

strongly correlated with the presence of the ALT phenotype (Schwartzentruber,

Korshunov, et al, 2012). Although a mechanism for how Daxx and ATRX cooperate to

cause ALT has not been proposed, it is logical to posit that their histone chaperone and

chromatin remodeling activities are intimately linked to this phenotype.

Daxx has also been implicated in a few scenarios of cancer using novel

pathways of action. One such instance is the interaction of Daxx with RAS-association

domain family protein 1A (Rassf1A). In human breast cancer cells with experimentally

reduced Daxx, the cells display resistance to taxol, a microtubule-destabilizing agent

commonly used as a chemotherapeutic. In Daxx-depleted cells, there is a prolonged

stabilization of cyclin B leading to faster progression through prophase but a lengthening

of prometaphase/metaphase, when Daxx and Rassf1A are found to interact. In cells that

respond to taxol therapy, taxol induces mitotic block creating micronucleated cells that

are incapable of proliferation. In Daxx- and Rassf1A-depleted cells, the accumulation of

cyclin B allows for cells to reenter mitosis after mitotic block is induced making them

resistant to taxol, whereas breast cancer cells expressing high levels of Daxx exhibit a

robust response to taxol (Giovinazzi, Lindsay, et al, 2012). Additionally, Daxx expression

is often upregulated in prostate cancer cells and is positively correlated with Gleason

score and metastasis. Daxx can induce chromosome instability in these cells via

inhibition of the anaphase-promoting complex (APC), leading to chromosomal polyploidy during prostate cancer development (Kwan, Lau, et al, 2013).

Scope of Dissertation

There is great ambiguity regarding the biology of Daxx in the cell. In this dissertation, I examine three distinct functions of Daxx that further elucidate the various processes in which Daxx is involved. First, I expand the previously found role of Daxx in p53 regulation by finding that phosphorylation of Daxx by ATM upon DNA damage leads to dissociation of the Daxx-Mdm2-Hausp complex in chapter two. Second, I uncover a novel role for Daxx in protein folding, whereby Daxx is able to refold and recover the activity of denatured substrates in chapter three. Finally, I briefly investigate if Daxx can regulate the cell cycle via a specific cyclin-dependent kinase (Cdk), Cdk6, in chapter four. The following sections of this chapter will provide brief introductions into the major biological themes of each of these projects: the mutual regulation between Mdm2 and p53, an overview of protein quality control in mammalian cells, and an outline of cell cycle progression.

The Mdm2-p53 Feedback Loop

The tumor suppressor p53 is one of the most extensively studied proteins as evidenced by the sheer number (over 60,000 as of 2012) of publications centering on its function and regulation. Its study has proven to be invaluable as more than 50% of human tumors harbor mutations in p53 itself or p53 effector proteins (Vogelstein, Lane, and Levine, 2000). Deletion of p53 in mice does not lead to embryonic lethality or developmental defects but does predispose the mice to spontaneous lymphomas and sarcomas by 2.5 months of age, leading to death by 6 months of age (Donehower,

Harvey, et al, 1992). 95% of malignant mutations of p53 are missense mutations found in the DNA-binding domain (DBD) (Greenblatt, Bennett, et al, 1994; Pavletich, Chambers, and Pabo, 1993). A proportion of these mutations lead to structural instability of p53 (Ang, Joerger, et al, 2006). It is known that mutant p53 often acts as a dominant-negative due to the formation of mixed tetramers, which contain both wild-type and mutant p53, abrogating the transcriptional effects of wild-type p53 (Chan, Siu, et al, 2004). A recent study suggests that structurally destabilized mutant p53 has greater aggregation propensity, which induces misfolding in wild-type p53 and thereby decreases the concentration of functional p53 in the cell (Xu, Reumers, et al, 2011).

In the cell, p53 levels are tightly controlled by its continuous ubiquitination and degradation, a process predominantly mediated by Mdm2 (Momand, Wu, and Dasgupta, 2000). In response to various stresses, such as DNA damage and oncogene activation, p53 is activated, which leads to the transcription of anti-proliferative genes. These effector proteins include pro-apoptotic (Bax and Apaf-1, e.g.), cell cycle arrest (p21, e.g.) or cellular senescence (p21 and PML, e.g.) promoting factors (Figure 1.3) (Vogelstein, Lane, and Levine, 2000). p53 is heavily post-translationally modified upon its activation, leading to conformational changes within the protein allowing for it to become a transcriptional activator. p53 is acetylated, methylated, ubiquitinated, and SUMOylated. The extent and type of modification depends on the cellular context (Gu, and Zhu, 2012). The N-terminus of p53 is extensively phosphorylated, including its phosphorylation by ATM and Chk2 at Ser15 and Ser20, respectively, upon DNA damage (Banin, Moyal, et al, 1998; Hirao, Kong, et al, 2000). One of the most important modifications that occurs on p53 is Mdm2-mediated ubiquitination (Gu, and Zhu, 2012).

Mdm2 is an E3 ubiquitin ligase not only for p53, but also for itself (Fang, Jensen, et al, 2000; Fuchs, Adler, et al, 1998). The Mdm2 gene was originally cloned from the
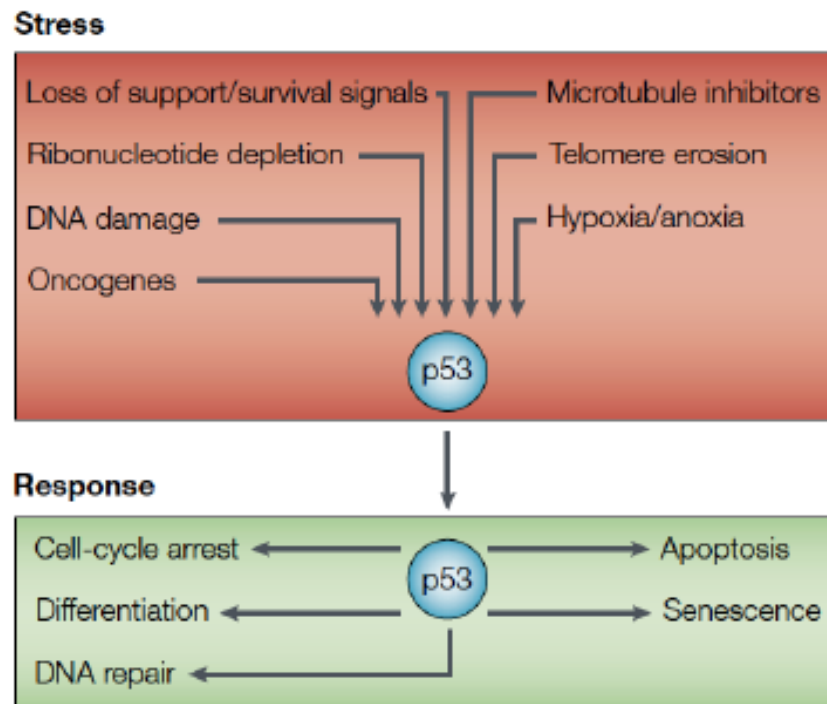
Figure 1.3. The p53 response.
Upon the induction of various stress signals, p53 levels are stabilized leading to the activation of different downstream regulators. The net effect of these regulators is to inhibit cell growth, ultimately preventing tumorigenesis. Reproduced with permission (Vousden, and Lu, 2002).

3T3-DM transformed mouse cell line, where it was gene-amplified on double-minute chromosomes (Fakharzadeh, Trusko, and George, 1991). Ensuing discoveries established the importance of Mdm2 as a p53 negative regulator not only via its ubiquitin ligase activity, but also due to its direct binding to the N-terminus of p53 and subsequent inhibition of p53 transcriptional activity (Chen, Marechal, and Levine, 1993; Kubbutat, Jones, and Vousden, 1997; Momand, Zambetti, et al, 1992). In an unstressed cell, Mdm2 associates with p53, targeting it for degradation (Figure 1.4). Upon the induction of DNA damage, for example, the Mdm2-p53 complex is disrupted through the accumulation of post-translational modifications on both p53 and Mdm2. Phosphorylation of Mdm2 by ATM inhibits its E3 ligase activity due to the disruption of higher-order Mdm2 oligomers that are necessary for its activity (Cheng, Chen, et al, 2009). The dissociation of p53 from Mdm2 allows p53 to avoid degradation and act as a transcriptional activator. Intriguingly, the Mdm2 gene is also a direct transcriptional target of p53, whereby Mdm2 levels increase in response to p53 transactivation (Figure 1.4) (Barak, Juven, et al, 1993; Picksley, and Lane, 1993; Wu, and Levine, 1997). The importance of the negative feedback loop between Mdm2 and p53 is underscored by the finding that though *mdm2* -/- mice are embryonic lethal, this lethality can be rescued via simultaneous deletion of the p53 gene (Jones, Roe, et al, 1995; Montes de Oca Luna, Wagner, and Lozano, 1995). This finding indicates that the main role of Mdm2 during development is to restrain the apoptotic activity of p53 and that a hyperactive p53 in incompatible with animal survival. Additionally, 7% of human tumors have gene amplification or aberrant expression of Mdm2 and Mdm2 overexpression and p53 mutations are generally mutually exclusive (Wade, Li, and Wahl, 2013). It is clear that the mutual regulation of p53 and Mdm2 is critical for cell viability as perturbations in this system can lead to human malignancies. Understanding the critical regulators of

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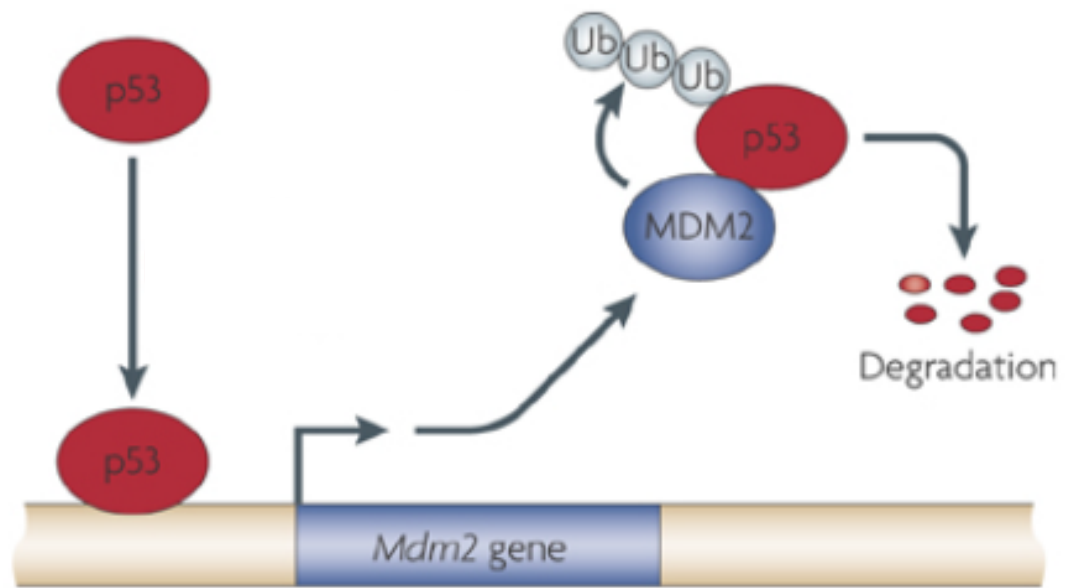
Figure 1.4. The p53-Mdm2 feedback loop.
In an unstressed cell, Mdm2 targets p53 for degradation in the 26S proteasome via ubiquitination. Upon the induction of stress, p53 is activated and transactivates *Mdm2*. Mdm2 then ubiquitinates p53 preventing further gene transactivation. This, in turn, forms a feedback loop between Mdm2 and p53. Reproduced and modified with permission (Murray-Zmijewski, Slee, and Lu, 2008).

these two proteins is essential and will be the focus of chapter two.

Protein Quality Control

The proper functioning of the cell relies on correct protein folding. There are approximately 20,000 proteins involved in the biological processes of the cell and for most of these the acquisition of a three-dimensional structure is a requirement for biological activity (Dobson, 2003). Protein folding in the cell can occur in various compartments. For example, during translation of proteins from the ribosome, protein folding occurs co-translationally as regions of the nascent polypeptide chain begin to fold before synthesis of the entire protein (Hardesty, and Kramer, 2001). Other proteins may undergo the majority of their folding in the cytoplasm after release from the ribosome, while others may fold in specific compartments such as the endoplasmic reticulum (ER) or mitochondria (Hartl, and Hayer-Hartl, 2002). During these folding processes, it is likely that portions of a nascent polypeptide will be exposed in partially folded, aggregation-prone states for considerable periods of time. Additionally, proteins folding during translation or in other compartments will be subject to the crowded intracellular milieu, strongly increasing the probability of non-native proteins to aggregate (Ellis, 2001; Ellis, and Minton, 2006). Moreover, errors in protein folding naturally occur due to genetic mutations or protein damage caused by cellular stress. Thus, the cell must maintain a proteostasis network to contend with these issues as the proper folding of a protein is not only important for its biological activity, but also for general cellular viability as the failure of proteins to fold properly is associated with proteinopathies such as neurodegenerative disorders, type 2 diabetes, cardiovascular disease, and cancer (Dobson, 2003; Hartl, Bracher, and Hayer-Hartl, 2011; Morimoto, 2008; Taylor, Hardy, and Fischbeck, 2002).

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How proteins achieve their native conformations has been the subject of research for many decades. The possible conformations a protein can adopt are astronomically large; thus, complex folding mechanisms are in place to promote the burial of hydrophobic residues and prevent aggregation (Hartl, Bracher, and Hayer-Hartl, 2011; Zwanzig, Szabo, and Bagchi, 1992). Current theories suggest that competing reactions of protein folding and aggregation exist in the cell and form a funnel-shaped potential energy landscape (Figure 1.5) (Dobson, 2003; Hartl, Bracher, and Hayer-Hartl, 2011; Kim, Hipp, et al, 2013). This theory postulates that there are few lower-energy, native-like conformations that a protein can have and that a protein travels on a downhill path toward a conformation of lowest energy (Dill, and MacCallum, 2012). As proteins move toward the native state, they form intramolecular contacts that create kinetically trapped intermediate conformations leading to free energy barriers that need to be overcome to reach the more favorable, low energy state. If many proteins are folding simultaneously in the same compartment, intermolecular contacts may form leading to the formation of amorphous aggregates, oligomers, or amyloid fibrils. Often times, the aggregated or fibrillar state may be most energetically favorable, though their formation is largely restricted *in vivo*. However, they may persist during times of stress or protein quality control failure (Dobson, 2003; Hartl, Bracher, and Hayer-Hartl, 2011).

Due to the complexity and significance of protein folding, the cell contains an elaborate protein quality control system to achieve proteostasis and maintain cellular health. Protein quality control is executed using an extensive network of proteins, the majority of which are molecular chaperones (Powers, Morimoto, et al, 2009). Although, the amino acid sequence is sufficient to confer three-dimensional structure to a protein,
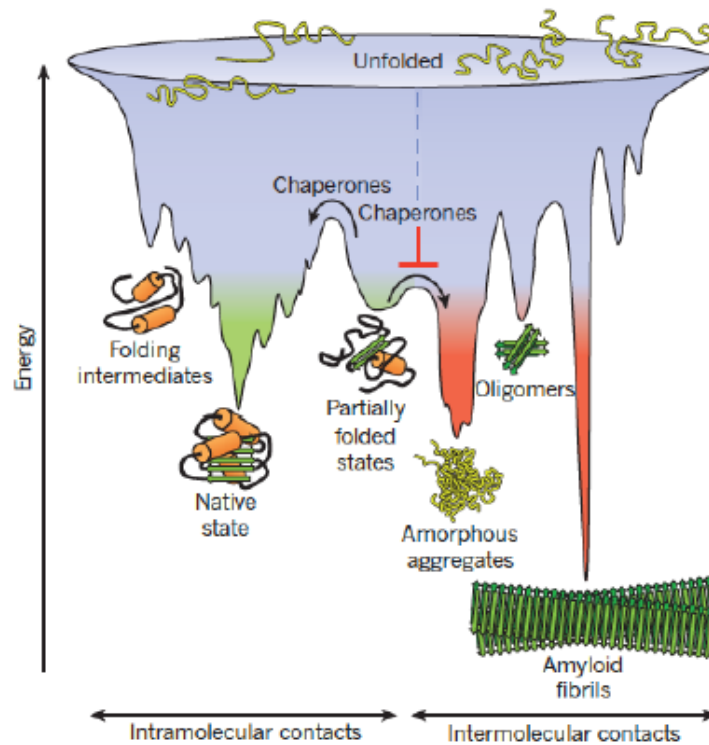
Figure 1.5. Energy landscape of protein folding.
Competing reactions between protein folding and aggregation naturally occur while a protein reaches its native structure. As proteins travel toward their native conformation, they form kinetically trapped intermediates that need to traverse energy barriers to reach their thermodynamically favorable conformation; steps made more efficient by molecular chaperones. Aggregates may form when inappropriate intermolecular contacts form during simultaneous folding of many proteins in the same compartments. *In vivo*, the formation of these structures is generally restrained by molecular chaperones. Reproduced with permission (Hartl, Bracher, and Hayer-Hartl, 2011).

many proteins require molecular chaperones to reach their native state efficiently (Anfinsen, 1973; Hartl, 1996). A molecular chaperone is defined as a protein that interacts with and helps another protein acquire its functionally active state without being present in its final structure (Hartl, 1996). To achieve proteostasis, molecular chaperones can promote the refolding of an aberrantly folded protein, send a misfolded protein for degradation, or sequester the protein to specialized quality control compartments (Figure 1.6) (Chen, Retzlaff, et al, 2011). For the purposes of this dissertation, I will focus on molecular chaperones and their folding activities.

Molecular chaperones interact with unfolded or partially folded protein subunits, for example, nascent chains emerging from the ribosome, where they participate in co-translational folding (Frydman, Erdjument-Bromage, et al, 1999; Hartl, and Hayer-Hartl, 2002; Netzer, and Hartl, 1997). The expression of molecular chaperones is often increased by cellular stress. Molecular chaperones can also stabilize non-native conformations and facilitate correct folding of protein subunits via coupling of ATP binding/hydrolysis to the folding process. *In vivo*, molecular chaperones strongly restrict the formation of aggregates and increase the overall efficiency of the protein folding process by reducing the probability of competing reactions, especially aggregation (Figure 1.5) (Dobson, 2003; Hartl, Bracher, and Hayer-Hartl, 2011).

One of the major classes of chaperones in the cells are the heat shock proteins (Hsps), so named because their expression is often upregulated upon the induction of cellular stress including heat shock, oxidative damage, and hypoxia, among others (Santoro, 2000; Whitley, Goldberg, and Jordan, 1999). The Hsps are named according to their molecular weight (in kDa): Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and the small Hsps (sHsps). The Hsps are involved in a multitude of protein quality control activities
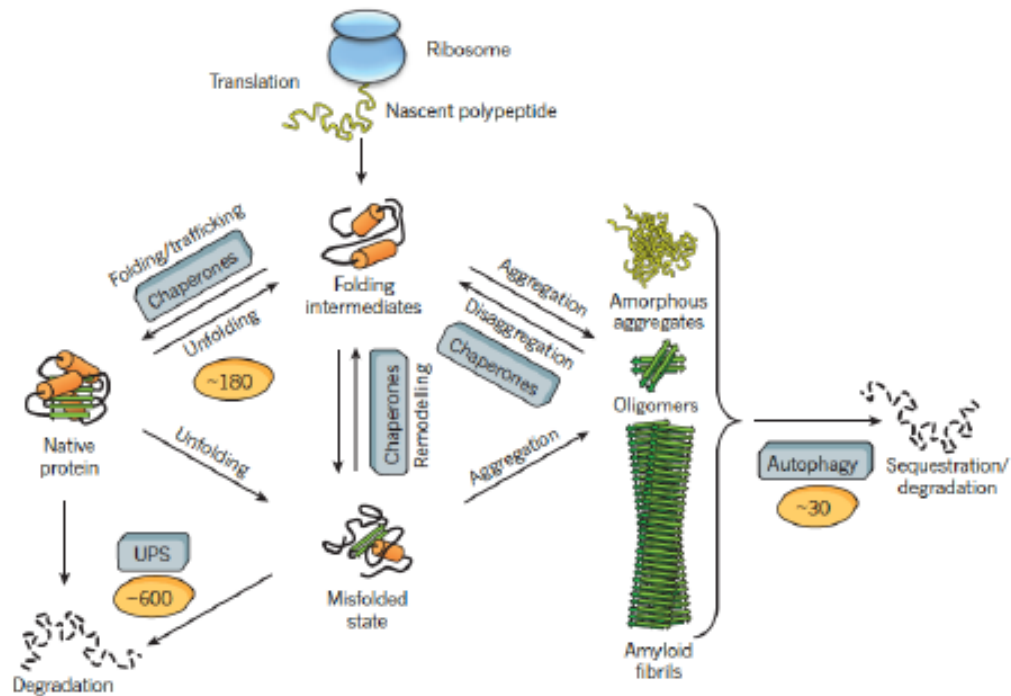
Figure 1.6. Chaperones and the proteostasis network.
The proteostasis network encompasses a large network of proteins, the majority of which are molecular chaperones. The chaperones are necessary to fold newly synthesized proteins, refold misfolded proteins, prevent protein aggregation, promote disaggregation, and send misfolded proteins for degradation via the proteasome or autophagy. Reproduced with permission (Hartl, Bracher, and Hayer-Hartl, 2011).

including *de novo* protein folding, refolding of stress-denatured proteins, signal transduction, and proteolytic degradation. The chaperones mainly involved in protein folding and refolding are the Hsp40s, Hsp70s, Hsp90s, Hsp100s and the Hsp60s, which are also known as chaperonins (Hartl, 1996; Hartl, Bracher, and Hayer-Hartl, 2011; Kim, Hipp, et al, 2013).

The function of the Hsp70 family of chaperones in protein folding can be described as the prevention of aggregation, the promotion of folding to the native state, and the solubilization and refolding of misfolded proteins (Mayer, and Bukau, 2005). The Hsp70 proteins have three main structural domains. The first is an N-terminal ATPase domain, which binds ATP and hydrolyzes it to ADP, a process catalyzed via association with Hsp40. The second is a substrate-binding domain that has affinity for the exposed hydrophobic regions on peptide segments. By binding to the hydrophobic patches on substrate molecules, Hsp70 and Hsp40 can prevent aggregation by blocking inappropriate interactions between non-native structures. Interestingly, the surface potential surrounding the substrate binding domain is negatively charged, which explains the contribution of positive charges in peptides for the affinity of Hsp70. Finally, the C-terminal portion is rich in α-helical structures, acting as lid for the substrate binding domain (Mayer, and Bukau, 2005). Hsp70 and Hsp40 can also assist non-native folding intermediates to fold into their native structures; however, the mechanism as to how this occurs is unclear (Mayer, and Bukau, 2005). Hsp70 also works in cooperation with the Hsp60 (chaperonin) class of chaperones to mediate protein folding. Chaperonins are large ring-like complexes that enclose substrates in their central cavity for folding (Horwich, Fenton, et al, 2007). Hsp70 and the chaperonins work together to aid nascent polypeptides in reaching their native structure; Hsp70 interacts with nascent

polypeptides as they emerge from the ribosome while the chaperonins act downstream to trap those substrates that escape the folding activity of Hsp70 (Frydman, Nimmesgern, et al, 1994; Langer, Lu, et al, 1992). Recently, an intriguing property of Hsp70 has emerged whereby, in an ATP-dependent manner, Hsp70 converts misfolded substrates into unfolded intermediates, which are then able to refold to their native state upon release from Hsp70. These results challenge the theory that Hsp70 chaperones are able to fold proteins; rather, they point toward an unfolding function prior to chaperone-independent refolding (Sharma, De los Rios, et al, 2010).

Despite the wealth of information regarding protein folding and misfolding, there is little known about how protein aggregation is reversed in the cell. It is known that the heat shock proteins can prevent aggregation, but very few possess disaggregation activity. The chaperonin, specifically GroEL-GroES in bacteria, and the Hsp70/40 systems have demonstrated *in vitro* disaggregation and reactivation ability in very specific cases using particular substrates: RNA polymerase (Skowyra, Georgopoulos, and Zylicz, 1990; Ziemienowicz, Skowyra, et al, 1993), malate dehydrogenase (Veinger, Diamant, et al, 1998) and glucose-6-phosphate dehydrogenase (Diamant, Ben-Zvi, et al, 2000). Most bacteria and eukaryotes including protozoa, fungi, and plants contain homologs of the AAA+ ATPase, Hsp104, a member of the Hsp100 class of chaperones. Hsp104 from yeast, in concert with Hsp70/40, can disaggregate and reactivate chemically denatured proteins *in vitro* (Glover, and Lindquist, 1998; Goloubinoff, Mogk, et al, 1999)*.* Hsp104 also confers thermotolerance to yeast cells; a property related to its ability to rescue misfolded or aggregated proteins (Sanchez, Taulien, et al, 1992). Hsp104 can cooperate with mammalian Hsp70/40 for disaggregation indicating that there is no species barrier between yeast Hsp104 and mammalian chaperones (Shorter,

2011). Strikingly, there is no metazoan homolog for Hsp104. Thus, it remains unclear if mammalian cells contain disaggregation abilities.

Recent studies have tried to determine if mammalian cells contain potent disaggregation activity and if this activity can be attributed to a single protein. Valosin-containing protein (VCP or p97), a member of the AAA+ ATPase family, has emerged as an attractive candidate. VCP is able to prevent the aggregation of denatured luciferase both *in vivo* and *in vitro* and it can also assist in the suppression and clearance of protein aggregates; however, its activity has not been clearly established (Kobayashi, Manno, and Kakizuka, 2007; Song, Wang, and Li, 2007). Recently, an Hsp70-related protein, Hsp110 has been shown to cooperate with Hsp70/40 in the mammalian cytosol to disaggregate and reactivate aggregated GFP and luciferase (Mattoo, Sharma, et al, 2013; Shorter, 2011). These results established that Hsp110, Hsp70, and Hsp40 constitute an ATP-dependent disaggregase machinery that is active in the mammalian cytosol and can act upon disordered, amorphous aggregates. Further, Hsp110, Hsp70, and Hsp40 can enhance the activity of Hsp104 in the disaggregation of amyloid fibers (Shorter, 2011).

All together, this evidence indicates that metazoan cells do possess disaggregation activity. Elucidation of the mediating factors in protein refolding and disaggregation would help in identifying new therapeutics for diseases caused by protein misfolding. Indeed, it has been shown that Hsp104, despite being a yeast protein, can be therapeutically efficacious in metazoan disorders such as Alzheimer's and Parkinson's disease, albeit in generally *in vitro* settings (Vashist, Cushman, and Shorter, 2010), underscoring the potential of molecular chaperones in ameliorating disease. In

chapter three, I examine the activity of Daxx in protein folding, potentially establishing it as a novel molecular chaperone.

The Mammalian Cell Cycle

Cell cycle regulation is crucial to the survival of the cell not only because it controls the growth of the cell, but also because it allows the cell time to detect and repair genetic damage. The mammalian cell cycle consists of four distinct phases—G1 (gap phase), S (synthesis), G2 (gap phase), and M (mitosis) phases (Figure 1.7). Cells that have temporarily or reversibly stopped dividing are said to enter a state of quiescence known as G0. G1, S, and G2 are collectively known as interphase, where the cell spends the majority of its time preparing for division via the acquisition of nutrients and replication of its DNA (Vermeulen, Van Bockstaele, and Berneman, 2003). The cell cycle is controlled by a number of checkpoints that facilitate the progression of the cell through the cycle ensuring that the cell is free of any defects in DNA synthesis or chromosome segregation (Malumbres, and Barbacid, 2009). The first checkpoint the cell encounters is the restriction (R) point. Once they pass this point, cells are able to proliferate without extracellular mitogenic stimuli (Pardee, 1974). The cell cycle is highly influenced by the proper expression of cyclins and their associated cyclin-dependent kinases (Cdks), which form specific complexes at each stage of the cell cycle to facilitate proper progression (Figure 1.7).

During G0 and G1 phase, the cell cycle is influenced by external mitogenic signals. Once activated by these extracellular signals, the D-type cyclins pair with their catalytic partners, Cdk4 and Cdk6, to promote progression through G1 phase (Sherr, and Roberts, 2004). The cyclin D-Cdk complexes work to promote cell division via the inactivation of two classes of cell cycle inhibitors: negative regulators of S-phase gene

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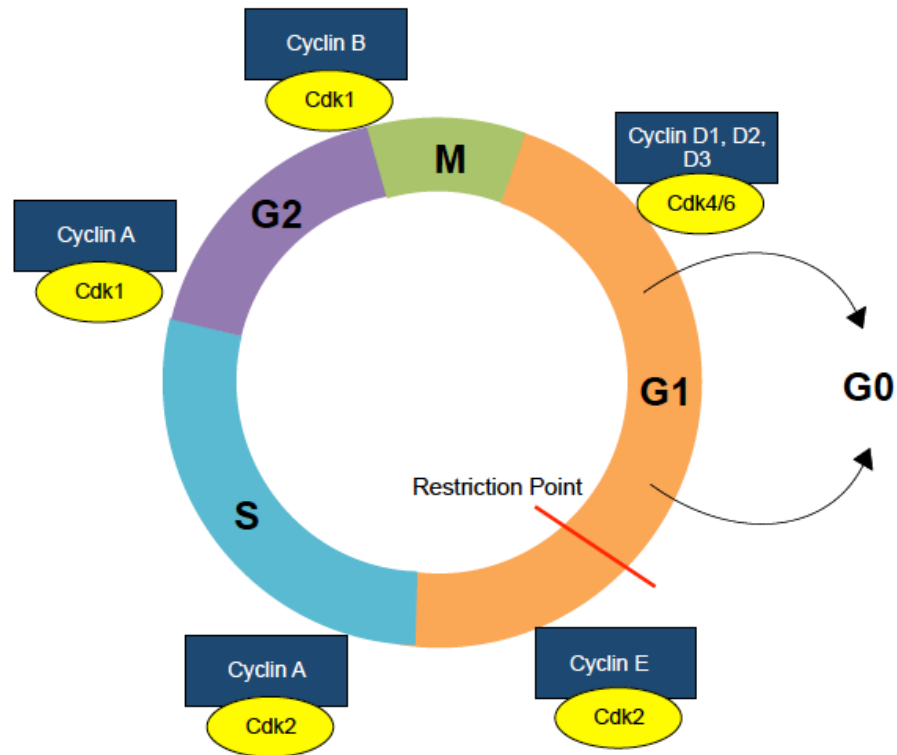
Figure 1.7. Schematic depicting the mammalian cell cycle.
The cell cycle is divided into four phases: G1 (first gap phase), S (DNA synthesis
phase), G2 (second gap phase), and M (mitosis). Cyclin-Cdk complexes regulate the
various stages of the cell cycle. G0 is a period of quiescence during which the cells have
temporarily or reversibly stopped dividing. The R denotes the restriction point at which
stage the cells do not require mitogenic signals to continue signaling.

expression and the Cip/Kip family of proteins (Sherr, and Roberts, 2004). Cyclin D-Cdk complexes are known to phosphorylate and functionally inactivate the retinoblastoma protein (pRb) and the pRb-related proteins, p107 and p130 (Harbour, Luo, et al, 1999). Inhibition of pRb leads to derepression of the E2F family of transcription factors, allowing transcription of a variety of proteins necessary for DNA metabolism and replication, ultimately allowing for progression into S phase. Additionally, the cyclin D-Cdk complexes are able to sequester p27 (Kip1) and p21 (Cip1) away from cyclin E-Cdk2 and cyclin A-Cdk2, relieving Cdk2 inhibition by p27 and p21 (Sherr, and Roberts, 1995; Toyoshima, and Hunter, 1994). If mitogenic signals are withdrawn, the cyclin D-Cdk complexes dissociate, allowing for mobilization of p27 bound to those complexes. Once freed from the complex, p27 is able to inhibit cyclin E-Cdk2 and promote exit from the cell cycle (Poon, Toyoshima, and Hunter, 1995).

The activation of E2F by the cyclin D-Cdk complexes during G1 leads to the transcription of cyclin E, allowing for the formation of cyclin E-Cdk2 complexes. This complex also phosphorylates pRb, allowing for further activation of E2F activity (Ohtani, DeGregori, and Nevins, 1995). pRb remains phosphorylated throughout the S, G2, and M phases and becomes dephosphorylated during the transition from M to G1 by PP1 (Ludlow, Glendening, et al, 1993; Nelson, Krucher, and Ludlow, 1997). The cyclin E-Cdk2 complex is at maximal activity at the transition between G1 and S phase and its activity significantly decreases at the beginning of S phase due to proteolytic degradation of cyclin E (Clurman, Sheaff, et al, 1996). Cyclin E-Cdk2 is responsible for the initiation of DNA synthesis and proper completion of S phase. As DNA replication continues, cyclin E levels decrease and the level of the mitotic cyclins (cyclins A and B) begins to increase (Sherr, and Roberts, 2004). Cyclin A-Cdk2 activity is first detected in late G1 phase and steadily increases as the cells begin to replicate their DNA, after

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which cyclin A is degraded before metaphase (Fung, and Poon, 2005). During S phase, the cyclin A-Cdk2 complex can phosphorylate substrates that start DNA replication from preassembled replication initiation complexes (Krude, Jackman, et al, 1997). After proteolytic degradation of cyclin A, cyclin B-Cdk1 complexes begin the transition into M phase (Mitra, and Enders, 2004). Activation of cyclin B-Cdk1 triggers its rapid accumulation in the nucleus and its levels continue to rise until the beginning of mitosis, after which they fall due to degradation of cyclin B. Activation of cyclin B-Cdk1 results in the breakdown of the nuclear envelope and the initiation of prophase (Hagting, Jackman, et al, 1999).

It is clear that the cell cycle is a carefully controlled process that utilizes cyclin-Cdk complexes for proper progression. However, this prevailing dogma has been challenged suggesting that not all these cyclins and Cdks are required for proper cellular proliferation. Genetic studies in mice have shown that mouse strains lacking each of the D-cyclins separately are mostly viable and display very narrow and restricted developmental abnormalities (Ciemerych, Kenney, et al, 2002; Fantl, Stamp, et al, 1995; Sicinska, Aifantis, et al, 2003; Sicinski, Donaher, et al, 1995). *Cyclin D1*-deficient mice are generally smaller in size and have severe hypoplastic retinas and pregnancy-insensitive mammary glands. Deletion of *cyclin D2* in mice leads to female sterility due to the inability of ovarian granulosa cells to proliferate normally in response to follicle-stimulating hormone (FSH). Male mice are still fertile but display hypoplastic testes (Sicinski, Donaher, et al, 1996). Finally, mice deficient in cyclin D3 have lymphoid abnormalities but are still viable (Sicinska, Aifantis, et al, 2003). Mice lacking all three cyclins appear normal until E13.5; however, they eventually die by E17.5 due to severe anemia and cardiac abnormalities. Interestingly, fibroblasts derived from these embryos were sustainable in culture albeit requiring higher mitogen concentrations to grow

(Kozar, Ciemerych, et al, 2004).

The necessity of the Cdks has also been studied and knockout mice lacking the various Cdks have been generated. *Cdk1 -/-* mice displayed arrested embryonic development at the blastocyst stage and MEFs lacking Cdk1 were unable to proliferate as the cells underwent premature senescence, suggesting that Cdk1 is essential for proliferation (Diril, Ratnacaram, et al, 2012). Conversely, deletion of *Cdk2* was shown to have no effect on mitosis in mice, but was important for the completion of prophase I during meiosis in both male and female germ cells (Berthet, Aleem, et al, 2003; Ortega, Prieto, et al, 2003). Mice deficient in Cdk4 and Cdk6 have also been generated; they are viable and show very narrow, tissue-specific defects (Kozar, and Sicinski, 2005). When only *Cdk4* is deleted, the mice have a reduced body size, are sterile, and become diabetic due to a reduced number of pancreatic β cells (Martin, Hunt, et al, 2003; Rane, Dubus, et al, 1999). *Cdk6 -/-* mice display defective hematopoiesis and lower cellularity in the thymus and spleen. The loss of Cdk6 also leads to delayed G1 progression in lymphocytes. Further observations suggest that Cdk6 is more essential for the expansion of differentiated populations rather than for proliferation of hematopoietic precursors (Malumbres, Sotillo, et al, 2004). The same study characterized double knockout *Cdk4;Cdk6 -/-* mice and demonstrated that embryos died beginning at E14.5 until the end of gestation due to severe anemia and major defects in multiple hematopoietic lineages. Many tissues still had normal proliferation rates and fibroblasts cultured from the double knockout embryos were able to cycle relatively normally.

The importance of the cell cycle proteins is underscored by the fact that their deregulation has been observed in various types of cancer. (Vermeulen, Van Bockstaele, and Berneman, 2003). In fact, overexpression of the D and E cyclins and Cdk4 are commonly observed in human tumors (Akli, and Keyomarsi, 2003; An,

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Beckmann, et al, 1999; Diehl, 2002; Donnellan, and Chetty, 1999; Yu, Sicinska, et al, 2006). It is possible that pharmacological disruption of these complexes may yield therapeutic efficacy for specific cancers. Yet, ambiguity exists regarding the necessity of these different proteins in cell cycle progression. Therefore, determining the cell-type specificity and regulation of these canonical cell cycle proteins is essential for understanding cellular proliferation. In chapter four, I discuss a novel function for Daxx as a potential regulator of the cell cycle associated protein, Cdk6.

CHAPTER 2: THE ROLE OF DAXX IN p53 REGULATION

<u>INTRODUCTION</u>

Tumor suppressors are necessary to prevent the proliferation of cells carrying cancer-causing alterations. These alterations can include the activation of an oncogene or DNA damage. The major responder to these cellular stresses is the tumor suppressor p53. Upon activation, p53 can induce apoptosis, cell cycle arrest, or senescence, amongst many other activities (Vogelstein, Lane, and Levine, 2000; Vousden, and Prives, 2009). Due to its potent anti-proliferative activity, p53 is highly constrained by the E3 ubiquitin ligase, Mdm2 (Haupt, Maya, et al, 1997; Kubbutat, Jones, and Vousden, 1997). In unstressed cells, p53 levels are maintained at a low level via ubiquitination by Mdm2; however, upon the induction of various stresses, Mdm2 stabilization is impaired and p53 levels are able to rise (Vogelstein, Lane, and Levine, 2000). Upon DNA damage, ATM is activated, leading to phosphorylation of p53 at Ser15 and at Ser20 by Chk2, a downstream kinase of ATM. These phosphorylation events cause stabilization of p53, partially via disruption of the p53-Mdm2 complex (Banin, Moyal, et al, 1998; Canman, Lim, et al, 1998; Shieh, Ahn, et al, 2000). Subsequent studies demonstrated that mutation of the phosphorylation sites in murine p53 do not robustly reduce p53 stabilization after DNA damage, indicating that other events are necessary for sustained p53 activation (Chao, Hergenhahn, et al, 2003; MacPherson, Kim, et al, 2004; Sluss, Armata, et al, 2004).

Our lab has previously shown that Daxx is a critical regulator of Mdm2 via the simultaneous association of Daxx with Hausp, a deubiquitinase, and Mdm2 in unstressed cells (Tang, Qu, et al, 2006). Thus, Daxx bridges Hausp to Mdm2, which deubiquitinates Mdm2 allowing for its stability and consequential p53 inactivation.

Induction of DNA damage leads to disruption of the Daxx-Mdm2-Hausp complex in an ATM-dependent manner allowing for p53 activation (Tang, Qu, et al, 2006). It is known that ATM phosphorylates Ser395 on Mdm2 and that this event interferes with the inhibitory effect of Mdm2 on p53 (Maya, Balass, et al, 2001). More recently it has been shown that ATM phosphorylates multiple sites in the C-terminus of Mdm2 upon DNA damage. These sites act redundantly to inhibit p53 degradation and stabilize p53 (Cheng, Chen, et al, 2009). When a phospho-mimic mutant of Mdm2 (S395D) was used to test its ability to associate with Daxx, it exhibited similar binding to Daxx as compared to wild-type Mdm2. This suggests that the Daxx-Mdm2-Hausp complex is potentially being regulated by additional phosphorylation events mediated by ATM. In the present study, we hypothesize that Daxx is an ATM target and that Daxx phosphorylation is important for its interaction with Mdm2 and consequential p53 activation. This study was recently published and demonstrated that Daxx is phosphorylated at Ser564 in response to DNA damage in an ATM-dependent manner (Tang, Agrawal, et al, 2013). Dr. Jun Tang identified the phosphorylation site on Daxx and raised the phospho-specific antibody to the aforementioned site. She also determined the ATM-dependence of this site *in vivo*. I performed the *in vitro* kinase assay demonstrating that ATM directly phosphorylates Daxx. I also executed subsequent analyses of Daxx phosphorylation regarding Mdm2 stability and p53 activation.

RESULTS

Daxx is phosphorylated at Ser564 by ATM in response to DNA damage

To determine if ATM phosphorylates Daxx during DNA damage, an antibody that recognizes the ATM substrate consensus sequence X-Ser/Thr-Gln (where X is a hydrophobic residue) was used to detect Daxx phosphorylation in human lung

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carcinoma (H1299) cells. The cells were transfected with Flag-Daxx and then treated with the genotoxic drug etoposide (ETP). Western analysis indicated that Daxx is phosphorylated soon after DNA damage induction and that this phosphorylation persists for at least eight hours (Figure 2.1). To map the phosphorylation site, residues from the N-terminus of Daxx were progressively deleted and these Daxx truncation mutants were transfected into H1299 cells treated with or without etoposide (Figure 2.2, A and B). Daxx phosphorylation was not detected for amino acids 570-740 but was detected in the other fragments, suggesting that the phosphorylation site resides within residues 340-570 of Daxx (Figure 2.2B). Analysis of this region revealed two potential ATM consensus sites: MAS^{424}QG and PVS^{564}QL. Both Ser424 and Ser564 were mutated to S424A and S564A and expressed in H1299 cells treated with or without etoposide. The S564A mutation, but not the S424A mutation, abolished Daxx phosphorylation upon treatment with etoposide indicating that S564 is the major ATM-targeted phosphorylation site on Daxx upon DNA damage (Figure 2.2C, lane 4 vs. 2 and 6). The Daxx Ser564 ATM consensus site is evolutionarily conserved across mammalian species, but not in fish or flies (Figure 2.3).

Phosphorylation of endogenous Daxx upon DNA damage

An antibody specific for the phosphorylated Ser564 residue on Daxx was made to confirm the modification of this site upon DNA damage (pS564-Daxx). This antibody detected phosphorylation of Daxx upon DNA damage in human osteosarcoma (U2OS) cells (Figure 2.4A, lane 2) and this signal was diminished when Daxx was knocked down using siRNA (Figure 2.4A, lane 4 vs. 2), underscoring the specificity of this antibody toward Daxx. To confirm that this antibody was recognizing phosphorylated Ser564, either wild-type, S564A, or S424A Daxx was expressed in H1299 cells and challenged
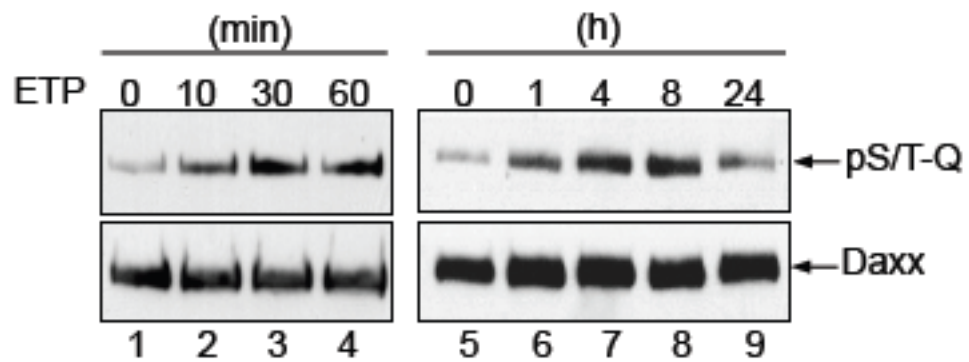
Figure 2.1. Daxx is phosphorylated upon DNA damage.
p53-deficient H1299 cells were transiently transfected with Flag-Daxx. 24 h later, the cells were treated with 10 μM etoposide (ETP) for the indicated durations. Cells were lysed and Flag-Daxx was immunoprecipitated with anti-Flag mAb beads and analyzed by western blot with antibodies against Daxx or phosphorylated ATM substrate consensus site (pS/T-Q). Performed by Dr. Jun Tang.
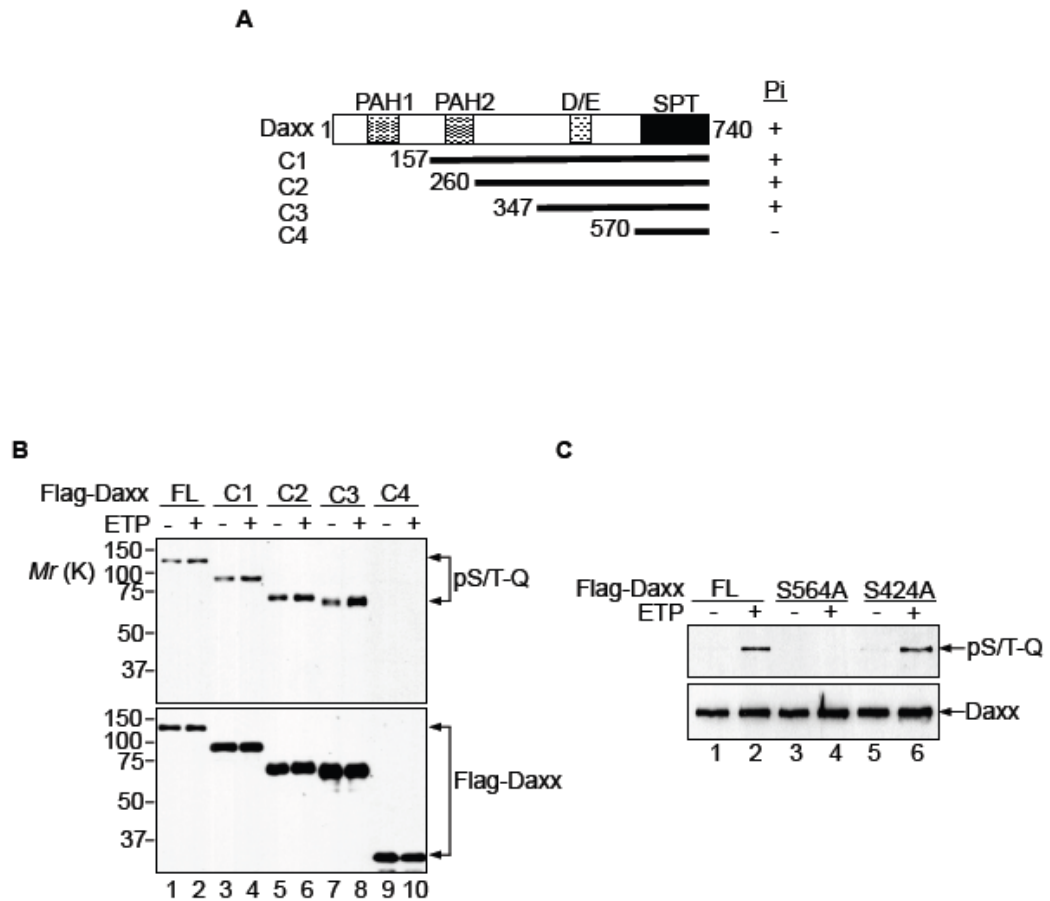
Figure 2.2. Daxx is phosphorylated at Ser564 upon DNA damage.
(A) Schematic representation of full length Daxx and its N-terminal deletion mutants. PAH, paired amphipathic helical domains. D/E, acid-rich region. SPT, Ser/Pro/Thr-rich domain. The amino acids in full length Daxx and in the N-terminus of each deletion mutant, and phosphorylation status (Pi) of these mutants are indicated. (B) H1299 cells expressing full-length (FL) Daxx and each of the deletion mutants were treated with 10 µM ETP for 1 h. Phosphorylation of these proteins was analyzed using Daxx and ATM substrate consensus site (pS/T-Q) antibodies. Exogenous Daxx phosphorylation existing before DNA damage was observed in some experiments, but not others. (C) Phosphorylation of Daxx, Daxx S424A, and Daxx S564A upon DNA damage was analyzed as in (B). Performed by Dr. Jun Tang.
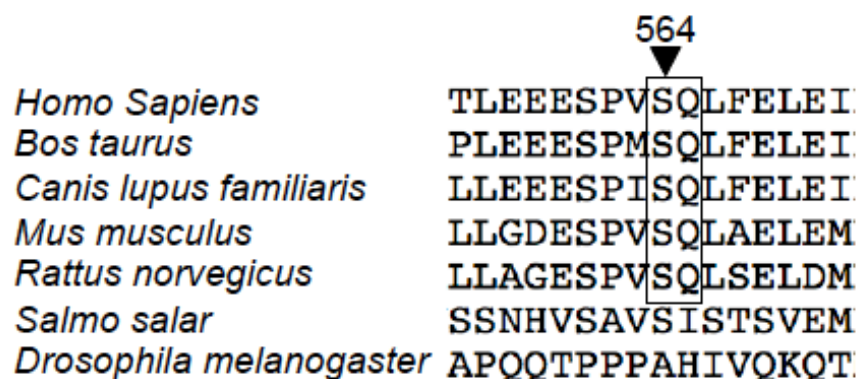
Figure 2.3. Ser564 of Daxx is evolutionarily conserved.
Alignment of the human Daxx (gi|48146287) sequence around Ser564 with the corresponding Daxx sequences from *Bos taurus* (gi|296474559), *Canis lupis familiaris* (gi|55956960), *Mus musculus* (gi|2253707), *Rattus norvegicus* (gi|18148939), *Salmo salar* (gi|148362139), and *Drosophila melanogaster* (gi|54144924). Alignment was run using Clustal W2 (Goujon, McWilliam, et al, 2010; Larkin, Blackshields, et al, 2007).
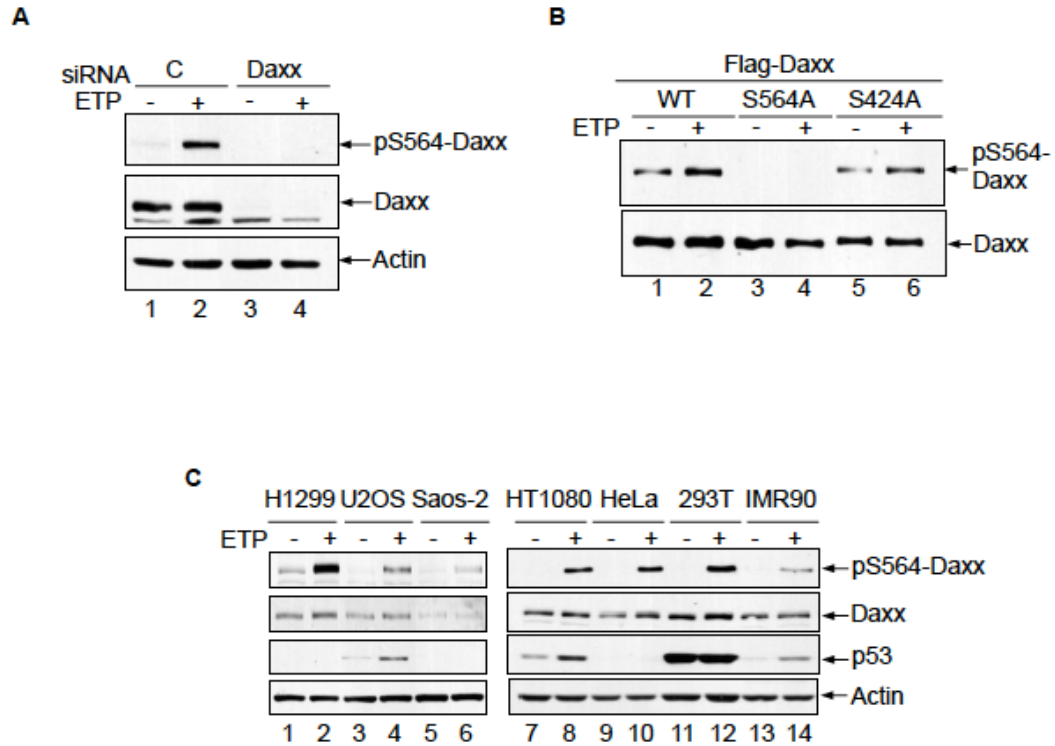
Figure 2.4. Phosphorylation of endogenous Daxx upon DNA damage.
(A) U2OS cells were transfected with control or Daxx siRNA and treated with 10 µM ETP
for 1 h. Cell lysates were analyzed by western blot using the phospho-specific Daxx
antibody, pS564-Daxx. (B) Western blot analysis of H1299 cells transfected with wild-
type (WT) Daxx, Daxx S564A, or Daxx S424A and treated with ETP for 1 h using pS564-
Daxx. (C) Phosphorylation of endogenous Daxx in multiple cell lines treated with and
without etoposide for 1 h. Cell lysates were analyzed using antibodies against pS564-
Daxx, Daxx, p53, and actin. Saos-2 (human osteosarcoma, p53 null); HT1080 (human
fibrosarcoma); HeLa (human cervix adenocarcinoma, p53 null); 293T (human embryonic
kidney); IMR90 (primary human lung fibroblast). Performed by Dr. Jun Tang.

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with etoposide. The phosphorylated Daxx band intensity was greatly decreased when Daxx S564A was expressed compared to wild-type or S424A Daxx (Figure 2.4B, lane 4 vs. lanes 2 and 6), further confirming the specificity of this antibody for phosphorylated Ser564. When this antibody was applied to cell lysates from a variety of cancer and primary cell lines, it was able to recognize DNA damage-induced Daxx phosphorylation in all cases, indicating the generality of this effect and its independence of p53 status (Figure 2.4C).

Daxx is phosphorylated by ATM *in vivo* and *in vitro*

To confirm the ATM-dependence of this phosphorylation event, *in vivo* and *in vitro* experiments were performed. First, H1299 cells were treated with ATM siRNA and etoposide. The lysates were analyzed with the phospho-specific antibody against Ser564 of Daxx, which showed that endogenous Daxx phosphorylation was diminished in ATM-depleted cells (Figure 2.5A, lane 4 vs. 2). To determine whether ATM directly phosphorylates Daxx at Ser564, we performed an *in vitro* kinase assay with various purified recombinant ATM and Daxx proteins in the presence of γ-^{32}P-ATP. Daxx was then analyzed by autoradiography, as well as by western blot using the anti-pS564-Daxx antibody. Daxx was phosphorylated *in vitro* by wild-type ATM, but not by a kinase-dead (KD) ATM mutant (Figure 2.5B, lanes 1 vs. 2). The small amount of pS564-Daxx signal in the reaction containing ATM KD (lane 2) was likely due to phosphorylation that pre-existed on the Daxx protein purified from HEK293 cells, because minimal ^{32}P incorporation of Daxx was detected in this reaction. Compared to Daxx, ATM-mediated phosphorylation of Daxx S564A was weakly detected by ^{32}P incorporation (lane 3), but not by anti-pS564 antibody (lane 4). This weak phosphorylation likely occurred on an amino acid residue(s) distinct from S564. Together, these data indicate that ATM can
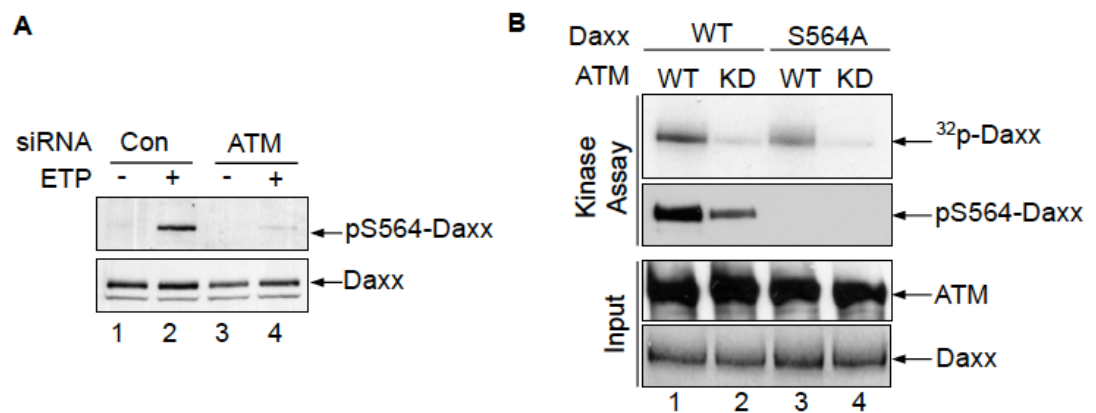
Figure 2.5. Daxx is phosphorylated by ATM *in vivo* and *in vitro*.
(A) H1299 cells treated with ATM siRNA or control siRNA were subject to DNA damage and analyzed for phosphorylation using pS564-Daxx antibody. Performed by Dr. Jun Tang. (B) *In vitro* kinase assay depicting Ser564 phosphorylation by ATM. Top two panels: phosphorylated Daxx was detected by autoradiography (^{32}P-Daxx) and western blot (pS564-Daxx). Bottom two panels: input of ATM and Daxx proteins were analyzed by western blot and Coomassie staining, respectively.

phosphorylate Daxx directly and mainly at Ser564.

Phosphorylation of Daxx at Ser564 regulates its interaction with Mdm2

Next, we sought to determine if phosphorylation of Daxx by ATM affected its interaction with Mdm2 and if this would have downstream consequences on the activation of p53. To determine if the binding of Daxx and Mdm2 was affected, we performed an immunoprecipitation where HA-Daxx or HA-Daxx S564A was co-transfected with Flag-Mdm2 in H1299 cells and Mdm2 was pulled down using anti-Flag beads. The co-immunoprecipitation assay showed that wild-type Daxx and Mdm2 readily dissociated from each other upon etoposide treatment; however, Daxx S564A continued to associate with Mdm2 during etoposide treatment, suggesting that the lack of Ser564 phosphorylation on Daxx enhances its interaction with Mdm2 (Figure 2.6A, lanes 6-8 vs. 3-5).

It is known that ATM can phosphorylate Mdm2 at Ser395 upon DNA damage (Maya, Balass, et al, 2001). We evaluated how the binding of wild-type and S564A Daxx would be affected with a phospho-mimic mutant of Mdm2 (S395D). Compared to wild-type Daxx, Daxx S564A was able to bind more strongly to Mdm2 S395D suggesting that a lack of phosphorylation at Ser564 on Daxx increases its ability to bind to phosphorylated Mdm2 (Figure 2.6B, lanes 3 vs. 2). Furthermore, a previous study reported that Daxx could be phosphorylated at Ser712 by the ATM/ATR signaling network (Matsuoka, Ballif, et al, 2007). To examine whether this phosphorylation site was involved in the Mdm2 interaction, a mutant form of Daxx was created (S712A) along with a double mutant where both the Ser564 and Ser712 were mutated to alanine (2SA). Daxx S712A displayed a similar binding affinity for Mdm2 S395D as wild-type Daxx (Figure 2.6B, lanes 4 vs. 2). Additionally, Daxx 2SA and Daxx S564A had

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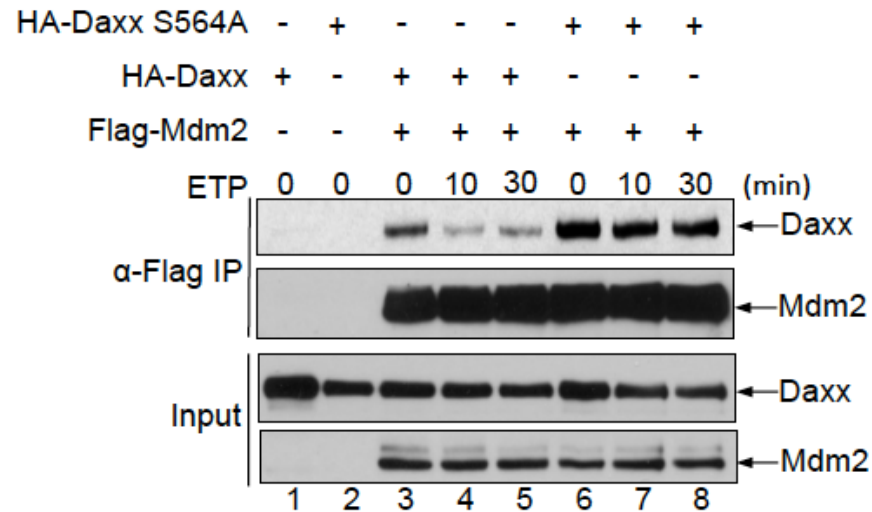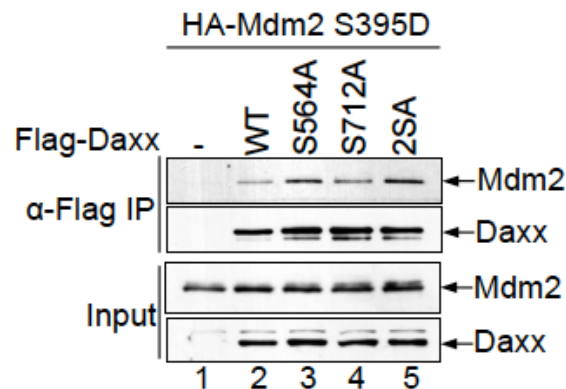
Figure 2.6. Phosphorylation of Daxx at Ser564 regulates its interaction with Mdm2.
(A) H1299 cells were transfected with either HA-Daxx or HA-Daxx S564A alone, or
together with Flag-Mdm2. Cells were treated with MG132 (20 μM) for 4 h and ETP (20
μM) for the indicated times. Cell lysates were incubated with anti-Flag mAb beads. Input
and immunoprecipitated proteins were analyzed by western blot using HA-HRP antibody
for Daxx and Flag-HRP antibody for Mdm2. (B) HA-Mdm2 S395D was transfected alone
and together with the indicated Flag-Daxx plasmids into *p53-/- Mdm2-/-* MEFs (mouse
embryonic fibroblasts). Cell lysates were incubated with anti-Flag mAb beads. The input
lysates and immunoprecipitated proteins were analyzed by western blotting.

indistinguishable interactions with Mdm2 S395D (Figure 2.6B, lanes 5 vs. 3). These results indicate that phosphorylation at Ser564 is the main event that disrupts the interaction of Daxx with phosphorylated Mdm2.

To determine if Daxx phosphorylation mediated the stability of Mdm2, we performed half-life analyses using the protein translation inhibitor cycloheximide. First, we expressed Mdm2 alone or with wild-type or Daxx S564A in H1299 cells. Wild-type Daxx was able to increase the protein expression and half-life of Mdm2 compared to when Mdm2 was expressed alone (Figure 2.7A, lanes 5-8 vs. 1-4) and Daxx S564A further enhanced the stability and half-life of Mdm2 (Figure 2.7A, lanes 9-12 vs. 5-8). To determine the effect of Daxx S564A on endogenous Mdm2, U2OS stable cell lines were established via retroviral transduction to express wild-type Daxx or Daxx S564A. The cells lines stably expressing Daxx S564A showed a greater ability to stabilize Mdm2 and increase its half-life, when compared to wild-type Daxx (Figure 2.7B, lanes 9-12 vs. 5-8). Finally, we tested whether this increase in half-life also occurred upon DNA damage. We expressed Mdm2 together with either Daxx or Daxx S564A. Etoposide was used to induce DNA damage after which cycloheximide was administered to the cells. Mdm2 stability was enhanced in the presence of Daxx, as expected (Figure 2.7C, lanes 5-8 vs. 1-4). Mdm2 stability was further enhanced when Daxx S564A was co-expressed, even though Daxx S564A expressed at a much lower level than wild-type Daxx (Figure 2.7C, lanes 9-12 vs. 5-8).

Our previous study indicated that Daxx is able to stabilize Mdm2 via association with Hausp (Tang, Qu, et al, 2006). To confirm that Daxx S564A-mediated stabilization of Mdm2 was also dependent upon Hausp, we treated U2OS cells with either Hausp or control siRNA and expressed increasing amounts of Daxx S564A. Endogenous Mdm2 stability is enhanced in the presence of Daxx S564A (Figure 2.8, lanes 2-4 vs. 1), but the
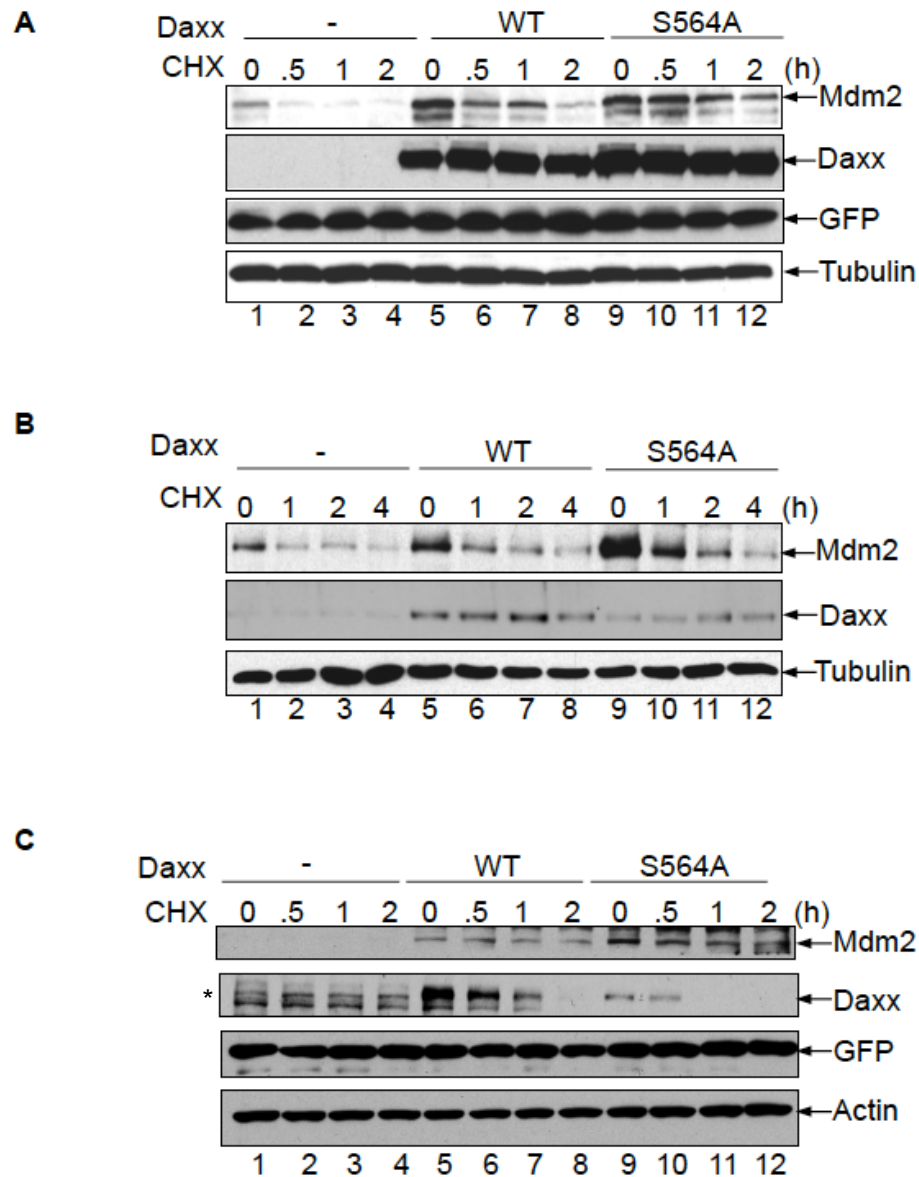
Figure 2.7. Phosphorylation of Daxx at Ser564 regulates the half-life of Mdm2.
(A) H1299 cells were transfected with HA-Mdm2 alone or with Flag-Daxx or Flag-Daxx S564A. Cells were treated with CHX (50 µg/ml) for indicated time and subjected to western blot analysis. Tubulin and GFP are shown as controls for sample loading and transfection efficiency, respectively. (B) Daxx S564A can prolong the half-life of endogenous Mdm2. U2OS cells stably expressing Flag-Daxx or Flag-Daxx S564A were treated with CHX (20 µg/mL) for the indicated times. Samples were analyzed using Mdm2, Daxx, and tubulin antibodies. (C) Daxx S564A can prolong the half-life of Mdm2 upon DNA damage. Flag-Mdm2 was transfected alone or together with the indicated HA-Daxx plasmids into *p53-/- Mdm2-/-* MEFs. Cells were treated with ETP (20 µM) for 1 h prior to cycloheximide (CHX, 50 µg/ml) treatment for indicated times. GFP and actin are shown as controls for transfection efficiency and sample loading, respectively. Asterisk indicates non-specific band.
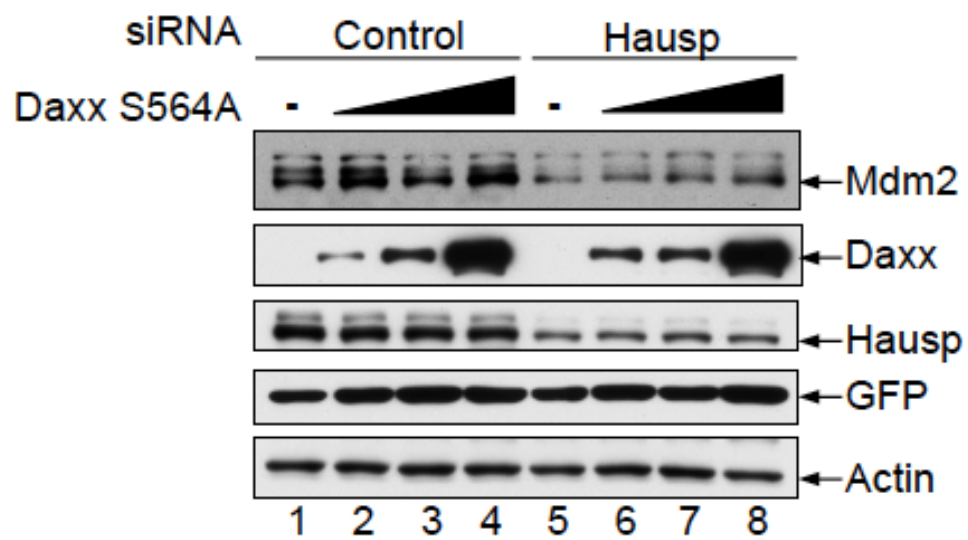
Figure 2.8. The effect of Daxx S564A on Mdm2 is dependent upon Hausp.
Increasing amounts of Flag-Daxx S564A were transfected into U2OS cells treated with either control or Hausp siRNA and analyzed by western blot. Actin and GFP are shown as controls for sample loading and transfection efficiency, respectively.

effect is greatly diminished in Hausp-silenced cells (Figure 2.8, lanes 5-8 vs. 1-4).

ATM-mediated phosphorylation of Daxx leads to p53 activation

We next examined if Daxx phosphorylation had consequences for p53 activation. Using human breast adenocarcinoma (MCF-7) cells, we stably expressed wild-type or S564A Daxx and assessed p53 levels. When Daxx S564A was present, there was an impairment of p53 activation upon etoposide treatment, presumably via greater stabilization of Mdm2 as compared to wild-type Daxx (Figure 2.9). The functional consequence of lower p53 levels was analyzed by measuring the protein (Figure 2.9A) and mRNA levels (Figure 2.9B) of the major p53 transcriptional target, p21. In both instances, p21 induction was weakened in the presence of Daxx S564A. These results indicate that ATM-mediated phosphorylation of Daxx upon DNA damage leads to Mdm2 instability and p53 activation.
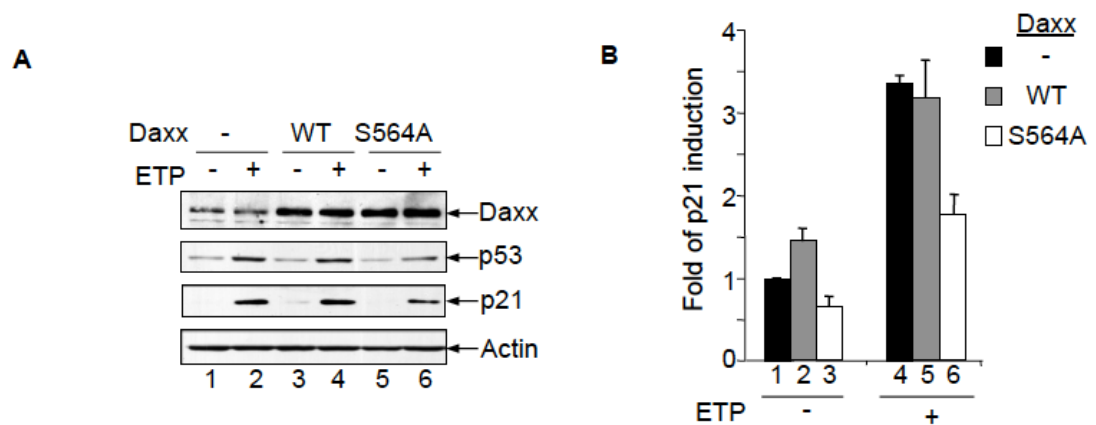
Figure 2.9. ATM-mediated phosphorylation of Daxx at Ser564 leads to p53 activation.
MCF-7 (A, done by Dr. Jun Tang) and U2OS (B) cells stably expressing Flag-Daxx or Flag-Daxx S564A were treated with 10 μM ETP for 8 h. Protein (A) and mRNA (B) expression was analyzed by western blot and quantitative RT-PCR.

MATERIALS AND METHODS

Antibodies and plasmids - Antibodies for the following proteins/epitopes were purchased from the indicated sources: actin (A2066), tubulin (T4026), and Flag (mouse monoclonal, M2, free (F3615) and conjugated to beads (A2220), and rabbit polyclonal (F7425)) (Sigma); ATM (Ab-3) and Mdm2 (Ab-1 and Ab-3) (Calbiochem); Daxx (M-112), p53 (DO-1), (Santa Cruz Biotechnology); phosphorylated ATM/ATR consensus site (X-pSer/Thr-Gln, where X is a hydrophobic residue) (#2851, Cell Signaling); GFP (JL-8, Clontech); Hausp/USP7 (A300, Bethyl Laboratories, Inc.); HA conjugated to horseradish peroxidase (Roche). Antibody specific to Phospho-Daxx Ser564 was made by Invitrogen using peptide PEELTLEEESPVpSQLFELEIEA. Plasmids encoding HA- or Flag-tagged Mdm2 and Daxx for transient transfection were made in pRK5, and plasmids encoding Flag-tagged Daxx for stable infection were made in the retroviral vector pBabe-puro. They were either previously described (Tang, Qu, et al, 2006), or generated for this study by PCR and confirmed by sequencing. ATM and ATM KD expression plasmids were kindly provided by Dr. M. B. Kastan.

Cell Culture - All cells were obtained from the ATCC. H1299 cells were grown in RPMI-40 media and all other cell lines in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For generating Daxx and control stable cell lines, retroviral constructs for Flag-Daxx and Flag-Daxx S564A, as well as the parental vector pBabe-puro, were separately transfected into either phoenix cells along with the retroviral packaging vector pCL-Ampho, or HEK293T cells along with pcgp (which encodes gag pol) and pHIT 456 (which encodes retroviral envelope). 48-72 h after transfection, the retrovirus-containing medium was used to infect U2OS or MCF-7 cells in the presence of 8 μg/mL polybrene. The infected cells were selected in the

presence of 2 µg/ml puromycin for 4-5 days.

Immunoprecipitation and western blot - Transfections were carried out using Lipofectamine 2000 (for DNA) or RNAiMAX (for siRNA) (Invitrogen) according to the manufacturer's instructions. siRNA sequence for Daxx was: CAG AAA CAT TAA TAA ACA ATA (Qiagen). siRNA sequence for Hausp was CCC AAA TTA TTC CGC CGG CAA A (Qiagen). 24 h after transfection, cells were lysed in IP lysis buffer (20 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.5% NP-40, 100 mM NaF, 1 mM PMSF, 1 mM DTT, 1X complete protease cocktail (Roche), and 10% glycerol). Samples were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Flag-Daxx or Flag-Mdm2 was immunoprecipitated with anti-Flag mAb beads and analyzed by western blot with anti-Daxx antibody (1:5,000), anti-phosphorylated ATM/ATR consensus site (pS/T-Q) antibody (1:500), anti-Flag antibody (mouse or rabbit, 1:5,000), or anti-HA antibody (1:5,000).

Kinase Assay - Flag-tagged ATM wild-type (WT), ATM kinase dead (KD), Daxx, and Daxx S564A were separately expressed in 293T cells and purified using M2 beads as previously described (Cheng, Chen, et al, 2009; Tang, Wu, et al, 2004). Daxx or Daxx S564A was incubated with ATM or ATM KD at 30°C for 1 h in kinase buffer (50 mM HEPES at pH 7.5, 150 mM NaCl, 20 mM $MnCl_2$, and 1% Triton X-100) containing 2 µM ATP and 10 µCi γ-^{32}P-ATP. Samples were fractionated on a 7.5% SDS-PAGE gel and detected by autoradiography and western blot.

Quantitative RT-PCR analysis - Total RNA was isolated from cells using TRIzol (Invitrogen). Reverse Transcription was performed using the First Strand cDNA

Synthesis Kit (Marligen Biosciences). A Taqman Gene Expression Assay (Applied

Biosystems) with validated human p21 (Hs00355782_m1) and 18s rRNA (4333760F)

primers/probe sets (Applied Biosystems) were used for qPCR and analyzed.

CHAPTER 3: DAXX AS A NOVEL MOLECULAR CHAPERONE

INTRODUCTION

Protein aggregation in cells arises due to the accumulation of misfolded proteins and leads to proteinopathies that can cause neurodegenerative diseases, diabetes, and cancer (Aguzzi, and O'Connor, 2010; Dobson, 2006; Xu, Reumers, et al, 2011). To contend with errors in protein folding, the cell maintains a sophisticated protein homeostasis network consisting of molecular chaperones to prevent aggregation or promote degradation of misfolded proteins (Dobson, 2003). Proteostasis is achieved using an extensive network of proteins, predominantly mediated via molecular chaperones (Hartl, Bracher, and Hayer-Hartl, 2011; Kim, Hipp, et al, 2013).

The Hsp70 class of molecular chaperones assists a wide range of folding processes including prevention of aggregation, promotion of folding to the native state, and solubilization of aggregated proteins (Mayer, and Bukau, 2005). Hsp70s work with the Hsp40 family of molecular chaperones to prevent aggregation through binding with hydrophobic patches on their substrate molecules, preventing inappropriate intermolecular associations, which could lead to aggregation (Mayer, and Bukau, 2005). Hsp70 chaperones can also promote the refolding of non-native proteins, yet the mechanism as to how this occurs is unclear. It is thought that via repetitive binding and release cycles of the substrate with Hsp70, the free concentration of the substrate is kept low to prevent aggregation and free molecules are allowed to fold to the native state (Mayer, and Bukau, 2005). Alternatively, Hsp70 may promote local unfolding of the protein through binding and release cycles, after which the protein can spontaneously fold into the native structure (Ben-Zvi, and Goloubinoff, 2001; Bukau, and Horwich,

1998; Slepenkov, and Witt, 2002). Hsp70 chaperones can also promote the solubilization of misfolded and aggregated proteins; however, this only occurs via association of Hsp70 with Hsp104, a member of the Hsp100 class of chaperones.

Intriguingly, there is no metazoan homolog for Hsp104 and to date, identification of a mammalian protein capable of renaturing large protein aggregates has remained elusive. Hsp104, a homolog of the AAA+ ATPase family, exists in the vast majority of bacteria and eukaryotes, but not in metazoan species (Doyle, and Wickner, 2009; Shorter, 2008; Vashist, Cushman, and Shorter, 2010). Hsp104 is able to rescue chemically denatured proteins after they have formed higher molecular weight aggregates, in combination with Hsp70 and Hsp40 (Glover, and Lindquist, 1998). It is unclear if animal cells possess protein disaggregases, but a potent mammalian disaggregating system has been discovered using a cell-free system, whereby Hsp110, a divergent relative of Hsp70, synergizes with Hsp70 and Hsp40 to confer protein disaggregase activity to the mammalian cytosol (Shorter, 2011).

Despite these discoveries, it is still unclear how Hsp70 promotes the refolding of non-native structures and how metazoan cells counteract large protein aggregates. We propose that Daxx may be a mediator of protein folding in mammalian cells for several reasons. First, Daxx has a diverse array of binding partners leading it to have seemingly disparate functions within in the cell (Salomoni, and Khelifi, 2006). This is similar to the Hsp70 proteins, in that they demonstrate broad substrate specificity shown through their ability to associate with the entire spectrum of heat-denatured proteins in *E. coli* (Mogk, Tomoyasu, et al, 1999). Second, Daxx contains an acid-rich region enriched in Glu and Asp acid residues between amino acids 414 and 505, constituting a polyanionic region. This region is reminiscent of the C-terminal portion of the smaller heat shock proteins,

without which their activity is diminished (Narberhaus, 2002). Additionally, polyanions such as nucleic acids and tubulin demonstrate *in vitro* chaperone activity (Guha, Manna, et al, 1998; Jones, Yazzie, and Middaugh, 2004; Rentzeperis, Jonsson, and Sauer, 1999). Further, the nucleoplasmin (NPM) family of proteins encompasses a group of nuclear chaperones that contain a conserved N-terminus within which lies a short acidic tract—a domain responsible for their oligomerization and chaperone function (Frehlick, Eirin-Lopez, and Ausio, 2007). These activities are most likely mediated by electrostatic interactions between the polyanions and client proteins. Finally, Daxx is a histone chaperone for the histone variant H3.3 that works with ATRX to assemble H3.3-containing nucleosomes (Drane, Ouararhni, et al, 2010; Elsaesser, and Allis, 2010; Elsasser, Huang, et al, 2012; Lewis, Elsaesser, et al, 2010). Its interaction with H3.3 has been mapped to a region within the central portion of Daxx, partially encompassing the acid-rich region (Drane, Ouararhni, et al, 2010). It is possible that its histone chaperone activity extends beyond deposition of H3.3 into chromatin; Daxx may also be able to assist H3.3 to attain its native structure. Additionally, it has been shown that Daxx depletion leads to accumulation of a soluble pool of newly synthesized H3.3 in the nucleoplasm, preventing H3.3 from localizing to PML-NBs (Delbarre, Ivanauskiene, et al, 2013). In their case, solubility refers to free H3.3 in nucleoplasm or cytoplasm. The idea that Daxx can associate with newly synthesized proteins is reminiscent of the co-translational folding activity of some of the molecular chaperones.

In the current study, we hypothesize that Daxx is a novel molecular chaperone. We found that Daxx has refolding activity toward denatured luciferase and wild-type p53. Daxx can also increase the solubility and activity of luciferase upon heat shock both *in vitro* and *in vivo*. Deletion mutant analysis reveals that not only is the acid-rich region of

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Daxx important for its protein folding activity, but also a paired amphipathic helical

domain near its N-terminus. These results indicate that Daxx is a candidate as a novel

molecular chaperone that has the ability to refold misfolded protein species. This activity

may also underlie the diverse set of proposed functions for Daxx.

RESULTS

Daxx can increase protein levels of luciferase in a transcription- and Hausp-independent manner

A commonly used substrate for the assaying of protein folding is luciferase.

Luciferase is highly thermolabile and its activity can be easily monitored using

luminescence. To examine the possibility that Daxx has an effect on luciferase

expression *in vivo*, we expressed Flag-NLS-luciferase (where NLS is a nuclear

localization sequence), Flag-Daxx, and GFP-Hsp70 in U2OS cells, as indicated (Figure

3.1A). Upon Daxx overexpression, luciferase protein levels increased in a dose-

dependent manner; however, luciferase levels were unchanged upon Hsp70

overexpression, as seen previously (Kampinga, Kanon, et al, 2003) (Figure 3.1A, lane 6

vs. lanes 3-5). To exclude the possibility that Daxx was affecting the transcription of

luciferase, Flag-Daxx was expressed with Flag-NLS-luciferase for both qPCR (Figure

3.1B) and western analysis (Figure 3.1C). Daxx overexpression had little effect on

luciferase gene expression, but did increase luciferase protein levels (Figure 3.1C, lanes

2 and 3).

Daxx is known to form a complex with Hausp, which leads to stabilization of

Mdm2 via the deubiquitinase activity of Hausp (Tang, Qu, et al, 2006). To examine if the
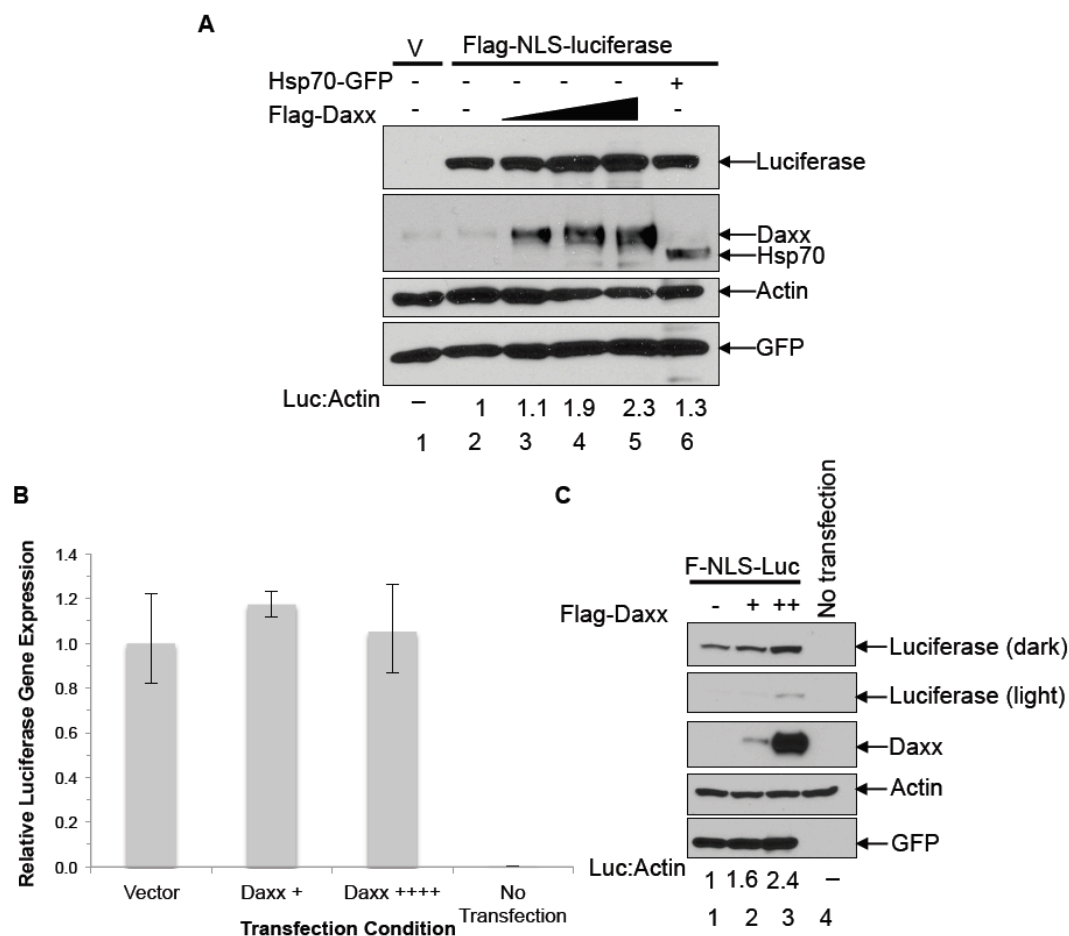
Figure 3.1. Daxx can increase protein levels of luciferase in a transcription-independent manner.
(A) U2OS cells were transfected with Flag-NLS-luciferase and increasing amounts of Flag-Daxx or GFP-Hsp70, as indicated. Cell lysates were analyzed by western blot. (B, C) The increase of luciferase expression is not dependent upon Daxx-mediated transcription. U2OS cells were transfected with Flag-NLS-luciferase and increasing amounts of Flag-Daxx concurrently for mRNA analysis via qPCR (B) and for protein analysis via western (C).

Daxx-Hausp interaction had a role in luciferase stabilization, we treated H1299 cells with Hausp or control siRNA and expressed increasing amounts of Daxx. The ability of Daxx to increase luciferase protein levels was unaffected by Hausp knockdown (Figure 3.2, lanes 5-6 vs. 2-3). These results show that Daxx can increase luciferase protein levels *in vivo* in a transcription- and Hausp-independent manner.

Daxx can renature heat-denatured luciferase *in vitro*

The observation that Daxx could increase luciferase protein levels in cells led us to posit that Daxx may have chaperone-like effects *in vitro*. To test this, we constructed a double-tagged Daxx with a glutathione-S-transferase (GST) tag at the N-terminus and a poly-histidine (6xHis) tag at the C-terminus. This Daxx fusion protein was purified from bacteria using a tandem-affinity approach followed by ion-exchange chromatography (Figure 3.3, A and B). To confirm the purity of Daxx, all bands that were visible by Coomassie staining were sent for mass spectrometry analysis (Figure 3.3B, lane 2). All bands were identified as Daxx, underscoring the purity of our purified Daxx. Upon confirmation that Daxx was pure, we performed traditional chaperone assays.

One characteristic of conventional chaperones, including those of the Hsp70/40 family, is their ability to prevent the denaturation of substrates. To perform this experiment, luciferase was concurrently incubated at 42ºC with GST and lysozyme as negative controls, Daxx (denoted as Daxx-His hereafter), and Hsp70/40 with ATP as a positive control. Daxx was unable to prevent loss luciferase of activity; however, the combination of Hsp70/40 was able to prevent denaturation of luciferase, as expected (Kubo, Tsunehiro, et al, 1999) (Figure 3.4A). For the same experiment when the Hsp70/40 data was removed from analysis, we were able to better see the kinetics of luciferase activity upon incubation with the other proteins. Strikingly, it seemed that Daxx
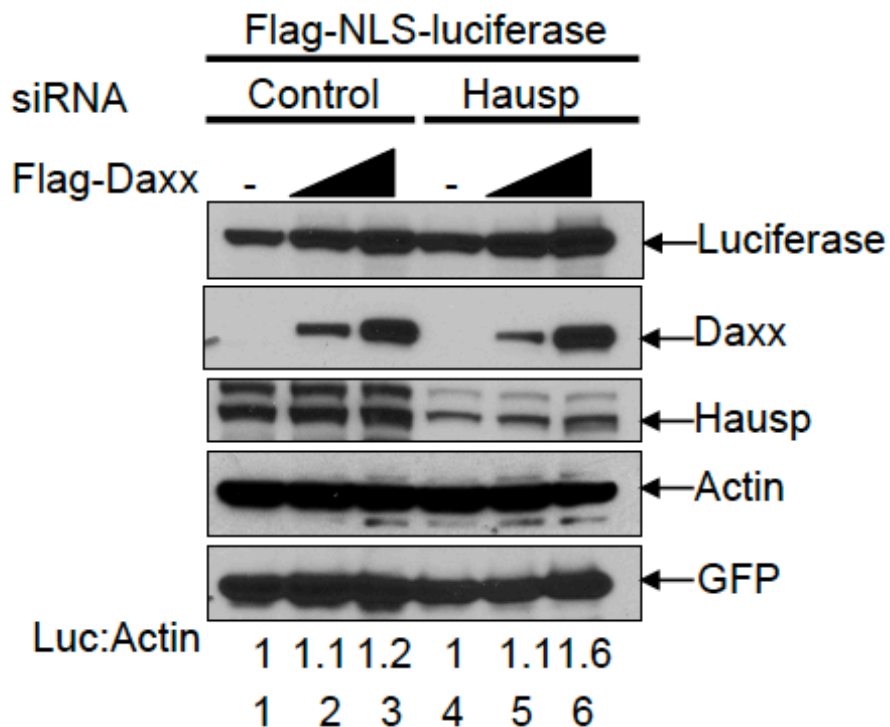
Figure 3.2. Daxx can increase protein levels of luciferase in a Hausp-independent manner.
Increasing amounts of Flag-Daxx were transfected with Flag-NLS-luciferase into H1299 cells. The cells were then treated with control or Hausp siRNA and analyzed by western blot. Actin and GFP are shown as controls for loading and transfection efficiency, respectively.

A

AcTEV protease site

| GST | Daxx | His |

≥ 20 L bacterial culture

GST batch purification

GST tag cleavage with AcTEV protease

His batch purification

Ion Exchange Chromatography

B

Daxx-His before IEX→

←Daxx-His after IEX

1   2
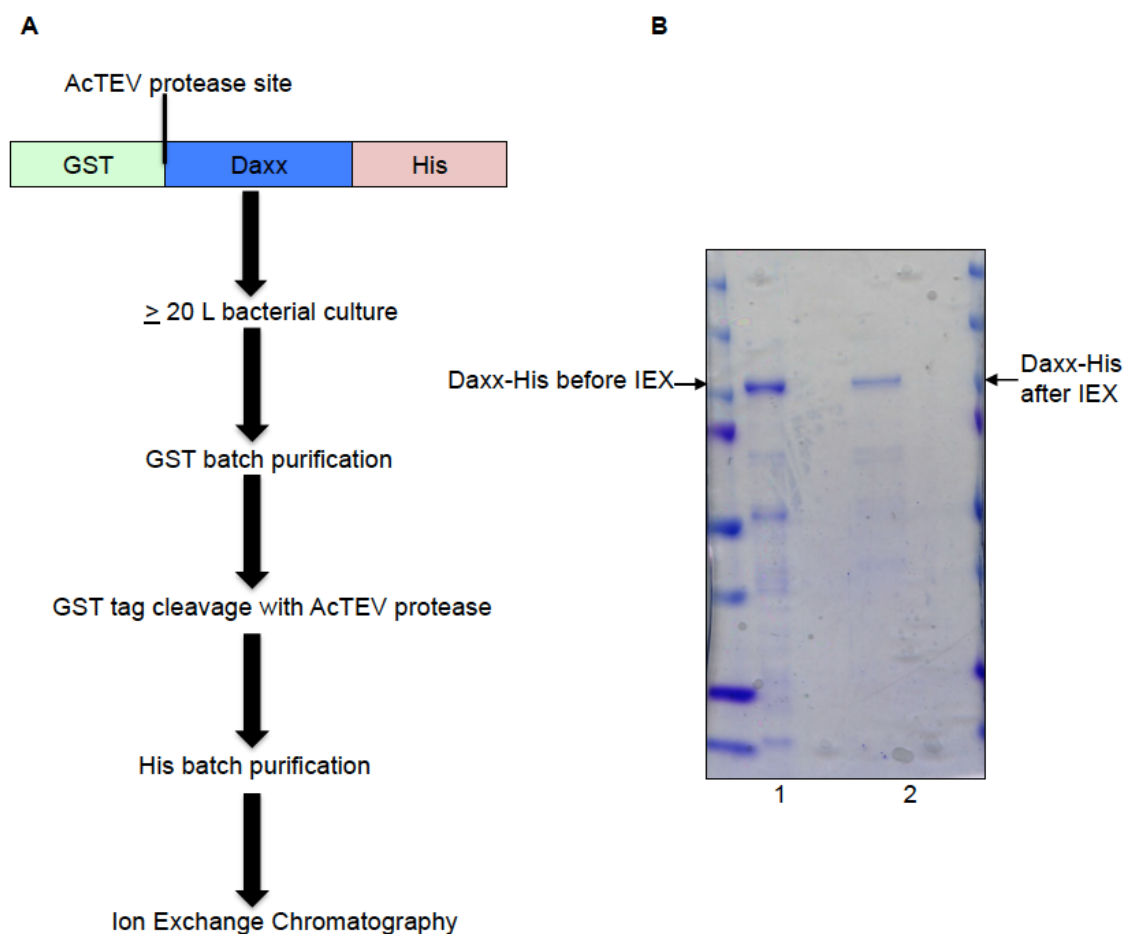
Figure 3.3. Protocol for Daxx purification.
(A) Schematic illustrating purification scheme when GST cleavage was required. When it was not required, that step was switched with protein elution using GSH. (B) Coomassie-stained gel depicting purity of Daxx-His before and after ion-exchange (IEX) chromatography.
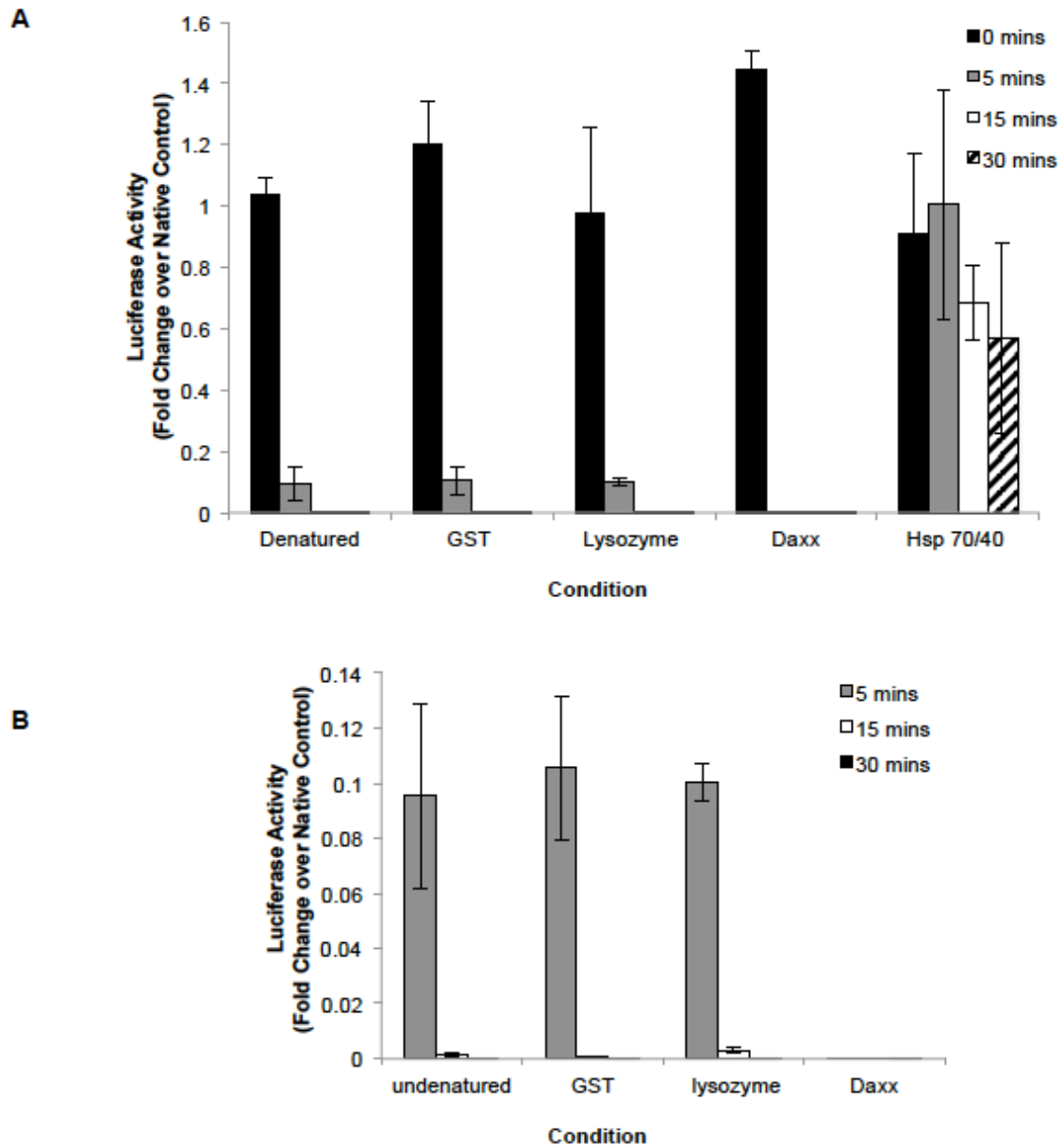
Figure 3.4. Daxx cannot prevent denaturation of luciferase *in vitro.*
(A) Heat-denatured luciferase (5 nM) was incubated with LRB or 50 nM GST, lysozyme, Daxx, or Hsp70/40 supplemented with 10 mM ATP at 42ºC. Luciferase activity readings were taken at the indicated times normalized to an undenatured control and represent the means $\pm$ SEM (n = 3). (B) Graph represents same data as in (A) except without Hsp70/40 and zero time point data.

was accelerating the denaturation of luciferase (Figure 3.4B), as luciferase had virtually zero activity by five minutes, whereas the other conditions retained approximately 10% of initial luciferase activity by five minutes. This result indicates that Daxx cannot prevent the denaturation of luciferase; rather, it accelerates luciferase denaturation.

Another characteristic of chaperones is that they are able to promote the refolding of denatured proteins. To assess this, luciferase was first denatured at 42ºC and then distributed to reactions containing buffer, lysozyme as a negative control, Daxx, or Hsp104/70/40 with ATP as a positive control. We used the Hsp104/70/40 system as a positive control as this has previously been shown to be effective in the renaturation of denatured substrates, such as luciferase and β-galactosidase (Glover, and Lindquist, 1998). Daxx was able to efficiently renature heat-denatured luciferase *in vitro* at 15- (Daxx-His +) and 25-fold (Daxx-His ++) excess of the luciferase concentration, albeit to a lesser extent than Hsp104/70/40 (Figure 3.5A). To further analyze the effect of Daxx on luciferase, a sedimentation assay was performed where denatured luciferase was incubated with buffer, lysozyme, or Daxx. The reactions were then centrifuged and separated into supernatant, containing soluble luciferase, and pellet, containing insoluble luciferase, fractions. The results indicate that Daxx was able to solubilize heat-denatured luciferase aggregates, as more luciferase was visible in the soluble fraction when Daxx was present (Figure 3.5B). Although with lysozyme it appeared that there was no luciferase in the pellet, lysozyme was not solubilizing luciferase, as it was not visible in the soluble fraction. This implies that the insoluble luciferase was part of an SDS-insoluble fraction that cannot be resolved using SDS-PAGE. Together, these results suggest that Daxx is not able to prevent the denaturation of luciferase, but it is able to promote the renaturation of an already denatured substrate.
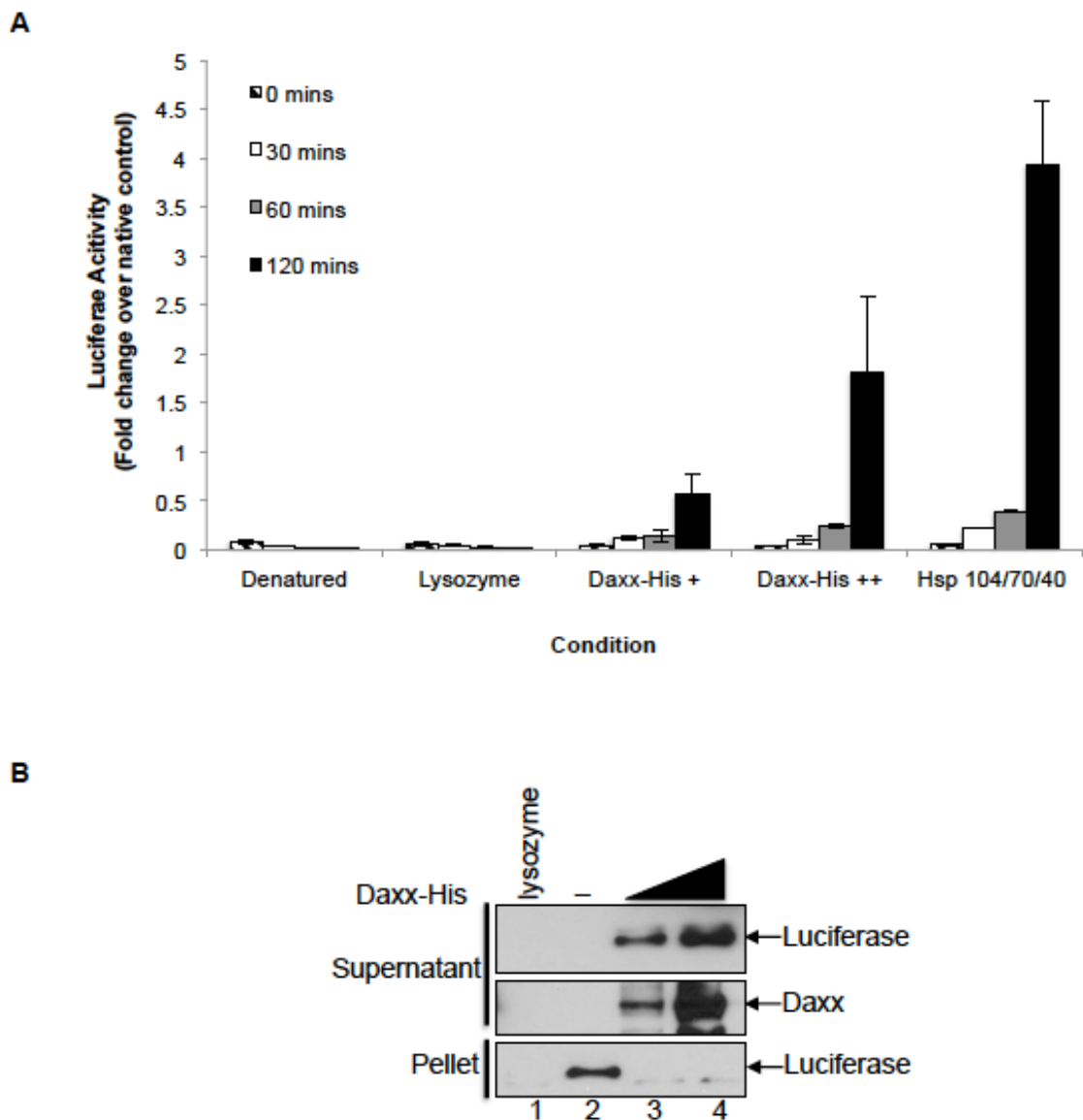
Figure 3.5. Daxx can renature heat-denatured luciferase *in vitro*.
(A) Heat-denatured luciferase (5 nM) was incubated with lysozyme (125 nM), Daxx (75 nM or 125 nM), and Hsp104/70/40 (125 nM) supplemented with 10 mM ATP at 25°C. Luciferase activity was read at the indicated times and expressed as fold change over undenatured control. Values represent means $\pm$ SEM (n = 3). (B) Heat-denatured luciferase (5 nM) was incubated with lysozyme (120 nM) and Daxx (30-120 nM) for 3 h at 25°C and then partitioned into soluble and insoluble fractions via sedimentation at 17,000 x g for 15 min. Fractions were analyzed via western blot.

To examine if Daxx had activity towards luciferase *in vivo*, we expressed Flag-Daxx, Flag-NLS-luciferase, and GFP-Hsp70 in H1299 cells and performed an *in vivo* luciferase assay (Michels, Kanon, et al, 1997) (Figure 3.6, A and B). In this assay, the cells were first treated with cycloheximide to prevent the translation of endogenous chaperone proteins upon heat shock. The cells were then heat shocked and allowed to recover at 37ºC. Although Daxx did not protect luciferase during heat shock, and in fact accelerated its loss of activity, it was able to recover it to a greater proportion (when compared to heat shock activity) than luciferase alone or when luciferase was expressed with Hsp70 (Figure 3.6A). The inability of Hsp70 to strongly recover luciferase activity was not unexpected. It has been shown that Hsp70 is not as effective in renaturation of luciferase in the nucleus as compared to the cytoplasm (Nollen, Brunsting, et al, 1999). Additionally, Daxx was also able to increase the protein levels of luciferase under control, heat shock, and recovery conditions (Figure 3.6B), confirming our earlier results (Figure 3.1A). This evidence suggests that Daxx cannot prevent the denaturation of luciferase; rather, it accelerates denaturation. It is then able to refold the denatured luciferase *in vivo* to a greater proportion than heat shock proteins.

Daxx can refold misfolded luciferase, but not higher molecular weight aggregates

On the basis that Daxx could renature heat-denatured luciferase, we sought to determine the physical properties of our luciferase substrate. To determine this, we used size fractionation to visualize the distribution of differentially formed aggregates via urea or heat denaturation. After denaturation, luciferase was applied to a gel filtration column for size fractionation. Fractions were collected and assessed for distribution using western blotting. When luciferase was denatured using heat denaturation, most of the luciferase eluted at later fractions, indicative of oligomeric, dimeric, and monomeric
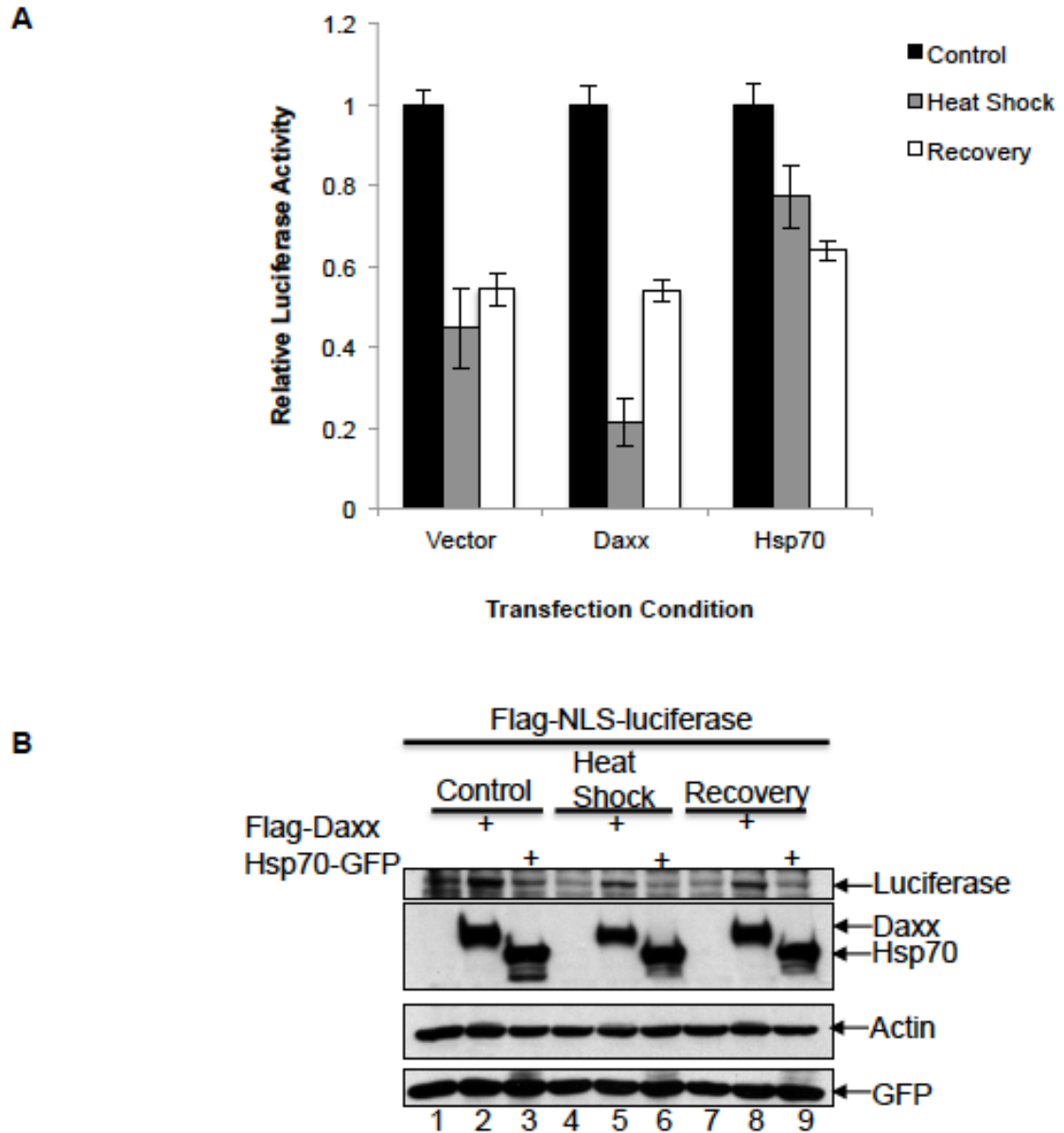
Figure 3.6. Daxx accelerates denaturation of luciferase and enhances recovery of luciferase *in vivo*.
(A, B) H1299 cells were transfected with luciferase and concurrently with either empty vector, Flag-Daxx, or GFP-Hsp70. Cells were then treated with CHX for 30 min to prevent translation of new chaperone proteins. Control samples were kept at 37°C for duration of assay while heat shock and recovery samples were placed at 45°C for 30 min, after which the recovery samples were placed back at 37°C for 3 h. (A) Luciferase activity is displayed relative to control samples. Values represent means $\pm$ SEM (n = 4, triplicate sampling). (B) Western analysis shows representative samples from each condition and experiment.
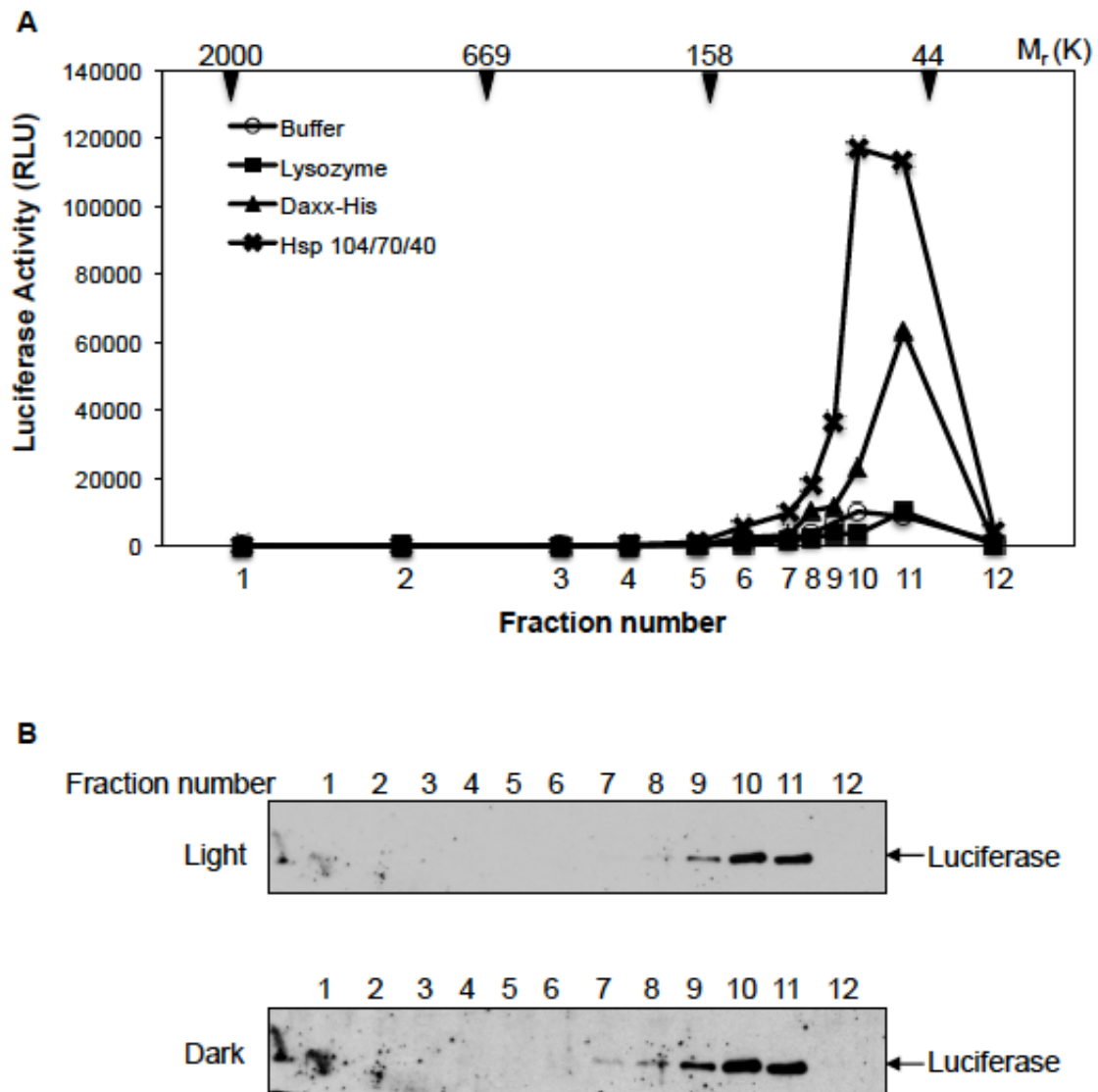
Figure 3.7. Daxx can renature heat-denatured luciferase.
(A, B) Heat-denatured luciferase (1 μM) was fractionated via gel filtration at 4°C. The elution profile was determined via western blotting (B). For refolding, column fractions in the range of 2000 kDa-44 kDa were incubated with LRB, or 100 nM lysozyme, Daxx, or Hsp104/70/40 supplemented with 10 mM ATP for 90 min at 25°C at which point luciferase activity was determined (A).

states (Figure 3.7, A and B). Selected elution fractions were then incubated with buffer, lysozyme, Daxx, or Hsp104/70/40 and luciferase activity was measured after 90 minutes of refolding. Daxx was able to efficiently renature luciferase when compared to lysozyme and the buffer control. The Hsp104/70/40 system had very strong reactivation ability, as expected (Glover, and Lindquist, 1998). However, when urea-denatured luciferase was size fractionated, Daxx was not able to disaggregate the higher molecular weight aggregates, which eluted at much earlier fractions than the heat-denatured luciferase (Figure 3.8, A and B). Hsp104/70/40 was able to efficiently renature the urea-denatured aggregates, as expected. These data indicate that Daxx can refold misfolded proteins, but it cannot disaggregate higher molecular weight aggregates.

The PAH2 and acid-rich domains of Daxx are important for its activity

Preliminary data indicated that the acid-rich and PAH2 domains of Daxx were most important for its activity toward luciferase. Based upon these findings, we constructed mutants that either did not express both the PAH2 (residues 157-260) and D/E (residues 414-505) domains (denoted as DaxxΔΔ), or only expressed the PAH2 or D/E domain (Figure 3.9, A-E). All of these Daxx mutants were constructed in the same vector as the full-length Daxx and purified using a similar method. When the GST tag was cleaved from the PAH2 and D/E domains, the proteins were unable to be purified. Thus, for those fragments we retained the GST tag and its activity was compared to full-length Daxx with an intact GST tag.

When DaxxΔΔ was assayed for activity in a luciferase renaturation assay (as in Figure 3.5), it was not able to renature heat-denatured luciferase (Figure 3.10A). When the D/E or PAH2 domain were tested alone, they were unable to efficiently renature

63
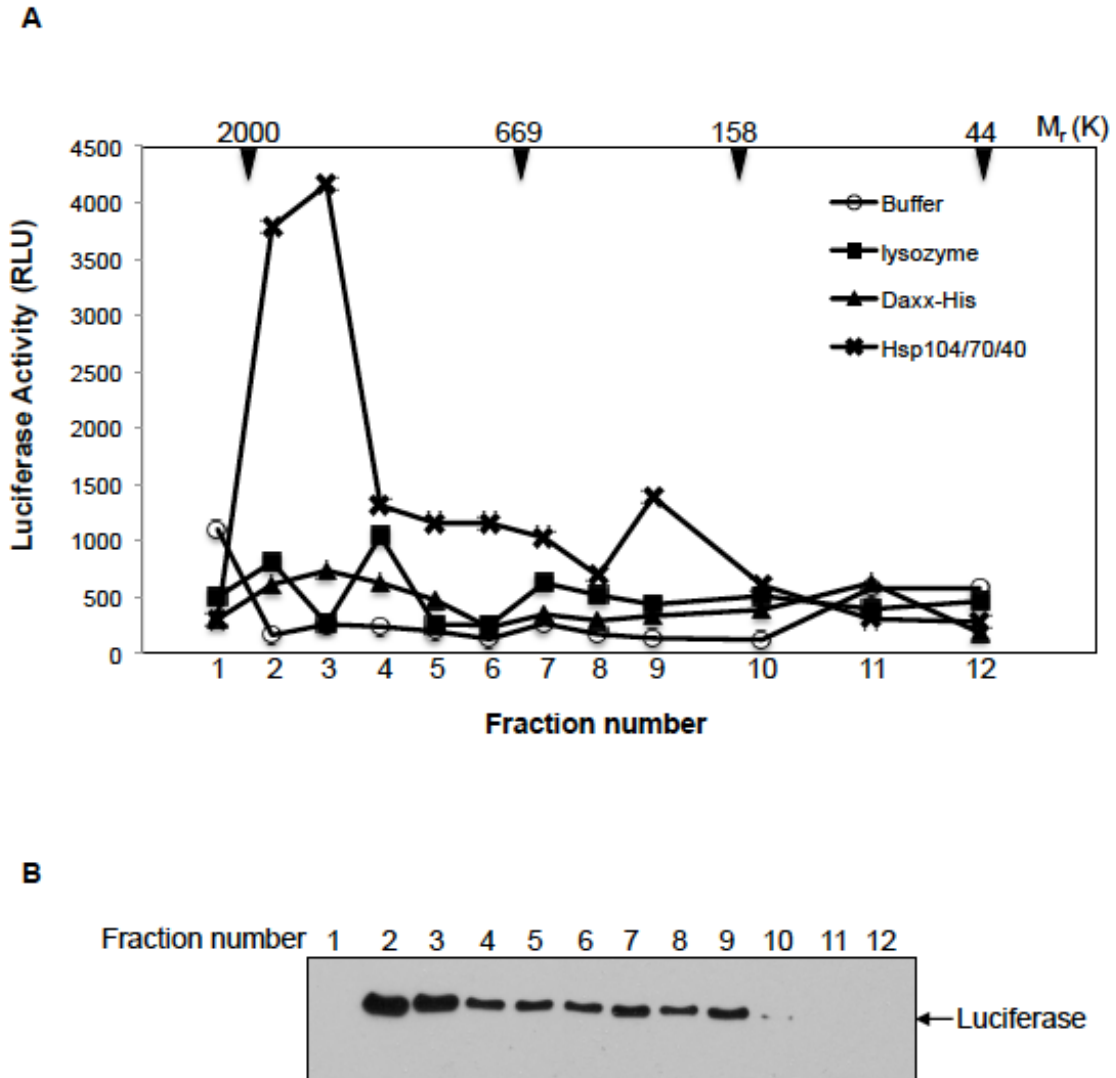
Figure 3.8. Daxx cannot renature higher molecular weight aggregates.
(A, B) Urea denatured luciferase (1 µM) was fractionated via gel filtration at 4ºC. The elution profile was determined via western blotting (B). For refolding, column fractions in the range of 2000 kDa-44 kDa were incubated with LRB, or 100 nM lysozyme, Daxx, or Hsp104/70/40 supplemented with 10 mM ATP for 90 min at 25°C at which point luciferase activity was determined (A).
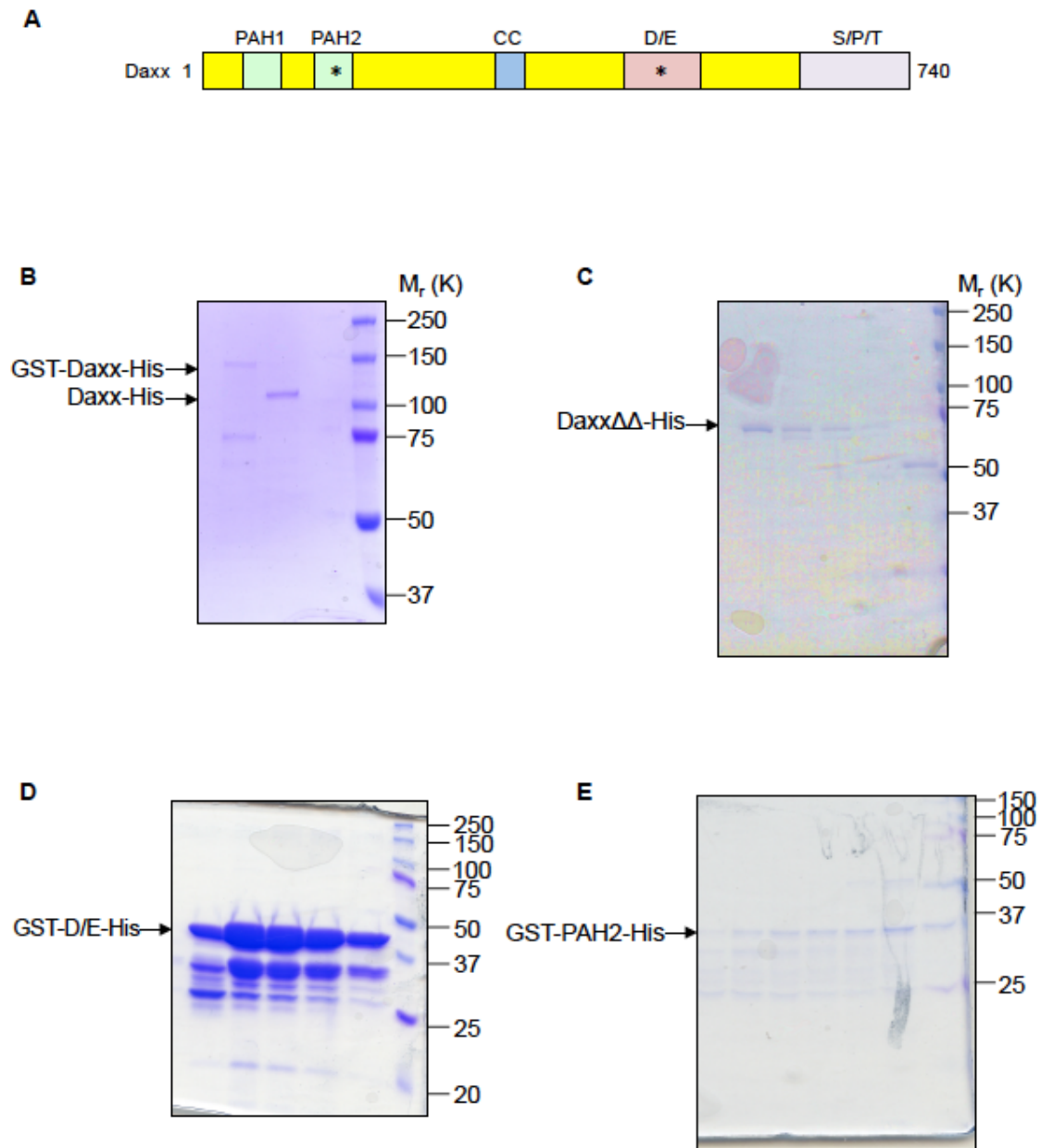
Figure 3.9. Purification of Daxx mutants.
(A) Schematic depicting structural domains of Daxx. PAH – paired amphipathic helices; CC – coiled coil domain; D/E – acid-rich region; S/P/T – serine/proline/threonine-rich region. Asterisks indicate deleted or isolated regions. (B-E) Coomassie-stained gels of proteins purified after tandem-affinity purification and IEX chromatography. For smaller fragments, GST-tag was retained due to loss of purification ability upon cleavage of tag.

A
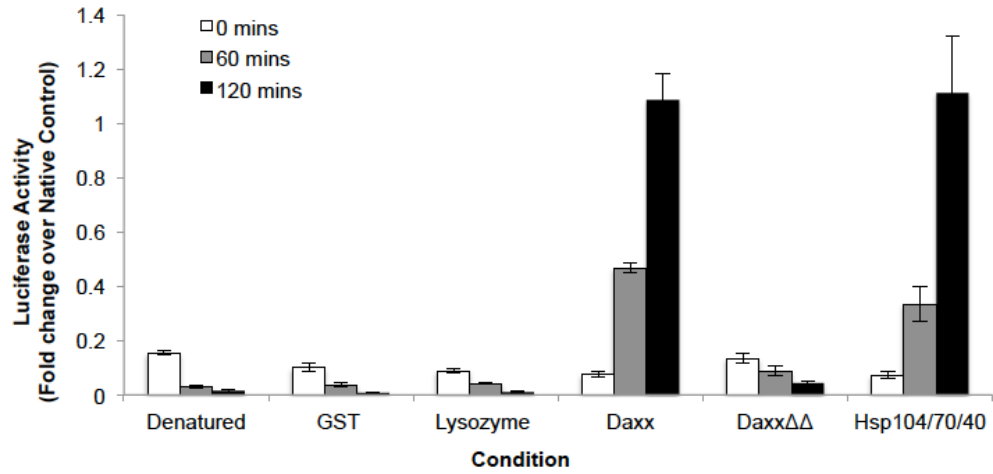
B
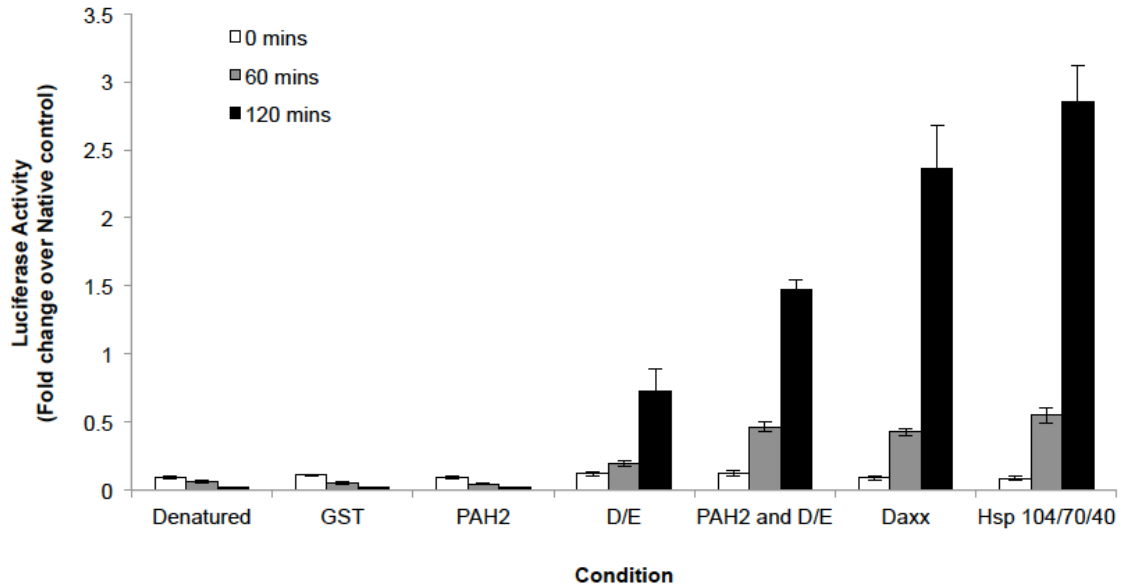
Figure 3.10. PAH2 domain and acid-rich region (D/E) are necessary for Daxx to renature heat-denatured luciferase *in vitro*.
(A) Deletion of PAH2 and D/E regions leads to loss of renaturation ability. Heat-denatured luciferase (5 nM) was incubated with 125 nM GST, lysozyme, DaxxΔΔ, Daxx, or Hsp104/70/40 supplemented with 10 mM ATP at 25ºC. (B) Mutants comprising only the PAH2 (250 nM) or D/E (250 nM) region were tested either alone or in combination for their ability to renature heat-denatured luciferase (5nM). For (B) and (C), luciferase activity was read at the indicated times and expressed as fold change over an undenatured control. Values represent means $\pm$ SEM (n = 3).

luciferase; however, when they were combined at equimolar ratios, there was a synergistic reactivation effect upon denatured luciferase (Figure 3.10B). This suggests that both domains are required for activity, but they are not sufficient on their own. To confirm that these domains and full-length Daxx were able to interact directly with luciferase, we purified Daxx, D/E and PAH2 fused only to GST. In an *in vitro* pull-down assay, immobilized GST-Daxx, -D/E, and -PAH2, but not GST alone, were able to pull down recombinant Flag-NLS-luciferase (Figure 3.11), indicating that full-length Daxx and the D/E and PAH2 domains are able to directly interact with luciferase.

Daxx preferentially acts upon misfolded luciferase

To examine *in vivo* if Daxx had any effect on misfolded luciferase, we examined whether Daxx could affect the protein levels of insoluble luciferase. Upon transfection of Flag-Daxx with Flag-NLS-luciferase in H1299 cells, the cells were treated with cycloheximide to inhibit new protein synthesis and the protein levels of insoluble luciferase were analyzed (Figure 3.12, A and B). As shown by densitometry analysis, the protein levels of insoluble luciferase decreased over time upon Daxx overexpression, indicating that Daxx was able to solubilize the insoluble luciferase. We were unable to detect a concomitant increase in the protein levels of soluble luciferase, most likely because the insoluble fraction comprises a very small portion of the total luciferase. Thus, any changes in the soluble fraction are not sensitive enough to be detected by western blot.

To determine if this effect translated to enhanced binding of Daxx to misfolded luciferase, a co-immunoprecipitation assay was performed. H1299 cells were transfected
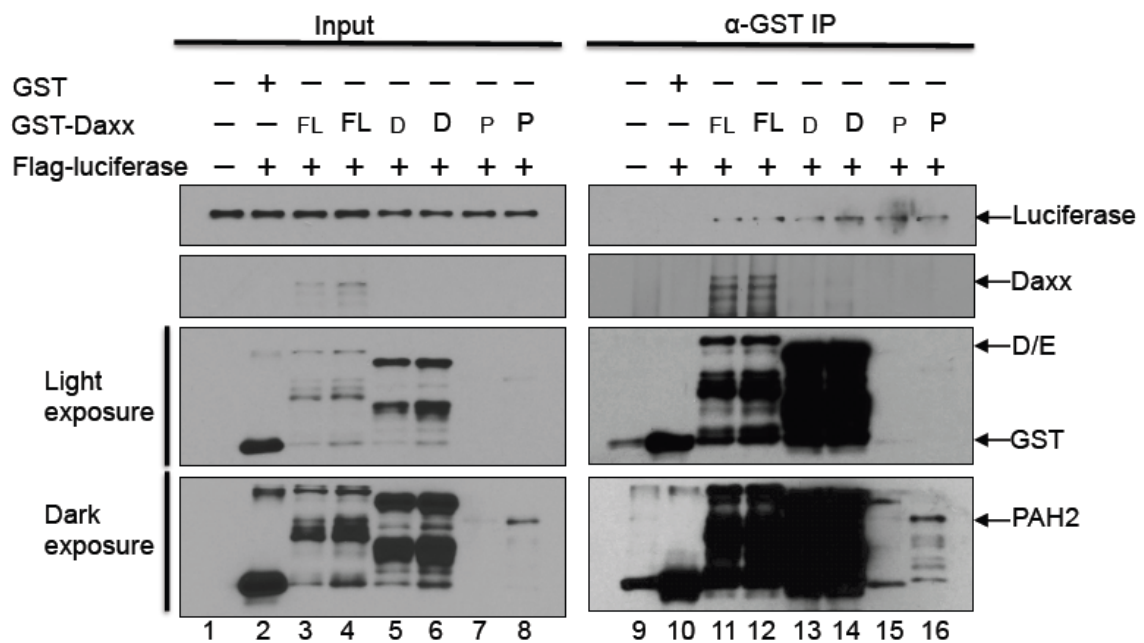
Figure 3.11. Daxx and Daxx fragments can bind to luciferase *in vitro*.
Recombinant Flag-NLS-luciferase purified from 293T cells was mixed with either lysis
buffer, GST, GST-Daxx, GST-PAH2, or GST-D/E purified from bacteria. Two different
amounts of Daxx and its mutants were used for immunoprecipitation. Input and IP were
analyzed by SDS-PAGE and western blot using anti-luciferase antibody for luciferase
and anti-GST antibody for GST, Daxx, and Daxx mutants. FL-full-length Daxx; D-acid-
rich region; P-PAH2 domain.

A
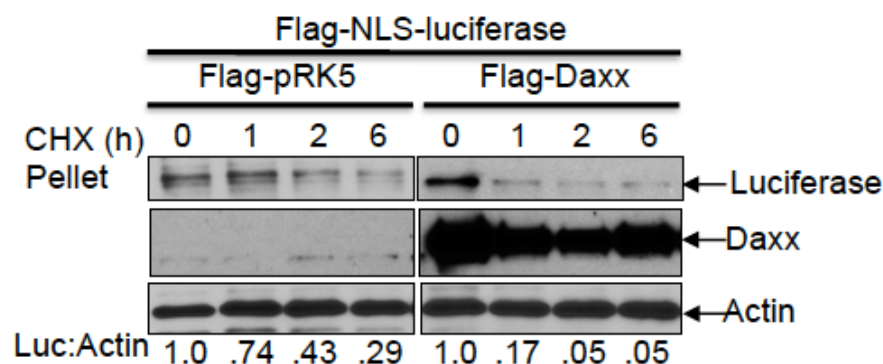
B
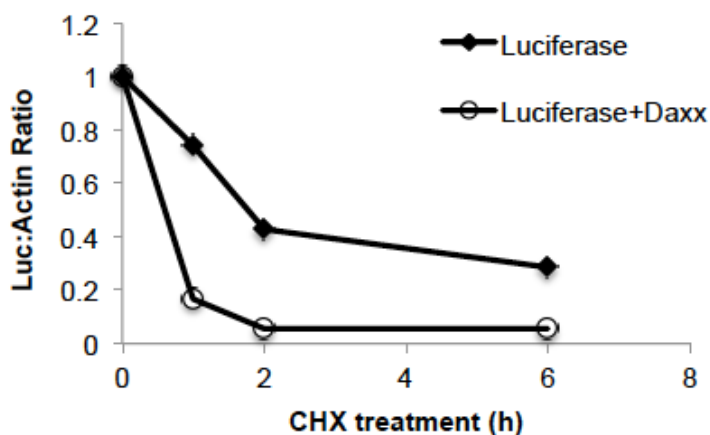
Figure 3.12. Daxx can decrease the protein levels of insoluble luciferase.
(A) H1299 cells were transfected with Flag-NLS-luciferase with or without Flag-Daxx.
The cells were treated with CHX (50 µg/mL) for the indicated times. Both the
supernatant and pellet fractions were collected; however, only the pellet fraction was
analyzed for luciferase. Samples were analyzed via western blot and using densitometry
analysis (ImageJ), the luciferase to actin ratio was determined and plotted as a function
of time (B).

with HA-Daxx with or without Flag-NLS-luciferase and subjected to heat shock as in Figure 3.6. Upon heat shock, more Daxx precipitated with luciferase than in the control condition (Figure 3.13A, lane 4 vs. 3). We confirmed that the heat shock of luciferase was effective by testing the activity of the samples containing luciferase prior to immunoprecipitation. The luciferase activity did decrease upon heat shock (Figure 3.13B). To further test this idea, we used an aggregation-prone luciferase mutant (Flag-nDMFluc-GFP) (Gupta, Kasturi, et al, 2011). When wild-type (Flag-nFluc-GFP) and mutant luciferase were immunoprecipitated, Daxx bound more strongly to the mutant luciferase, even though it precipitated less effectively than the wild-type luciferase (Figure 3.14). These results suggest that Daxx preferentially binds to misfolded luciferase.

Daxx can solubilize p53 *in vitro* and *in vivo*

Since luciferase is not found in human cells, we sought to find a physiologically relevant target of Daxx. It has been established by us and other groups that Daxx can regulate p53 (Gostissa, Morelli, et al, 2004; Tang, Qu, et al, 2006; Tang, Agrawal, et al, 2013; Zhao, Liu, et al, 2004). We repeated a similar experiment to Figure 3.5B; we purified Flag-p53 from mammalian cells and denatured it at 37ºC for 3-4 min to induce misfolding (Figure 3.15A). Upon addition of increasing amounts of Daxx, p53 was solubilized and shifted to the supernatant fraction (Figure 3.15A, lanes 3-6 vs. 1). The addition of lysozyme seemed to increase the misfolding of p53 as more p53 was found in the pellet fraction, similar to our result from Figure 3.5B and it was not able to solubilize p53 as p53 was not detected in the supernatant fraction. To test if Daxx could also stabilize p53 *in vivo*, we utilized *p53 -/- Mdm2 -/-* MEFs to eliminate the effect Daxx has on Mdm2. When Flag-Daxx was transfected in increasing amounts with steady
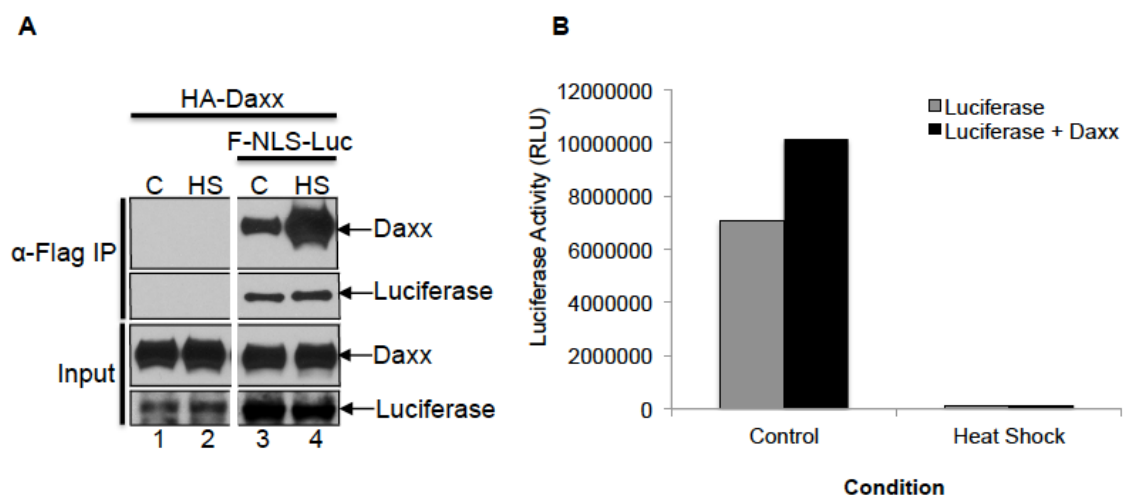
Figure 3.13 Daxx can preferentially associate with heat-denatured luciferase.
(A, B) Daxx preferentially binds to luciferase upon heat shock. H1299 cells were transfected with HA-Daxx and Flag-NLS-luciferase as indicated. Cells were then treated with CHX (50 µg/mL) for 30 min. Control samples were kept at 37°C for the duration of the assay while heat shock and recovery samples were placed at 45°C for 30 min, after which the recovery samples were placed back at 37°C for 3 h. Cell lysates were incubated with anti-Flag M2 beads and input and IP samples were analyzed by western blot (A). Luciferase activity was determined for samples with luciferase prior to immunoprecipitation (B).
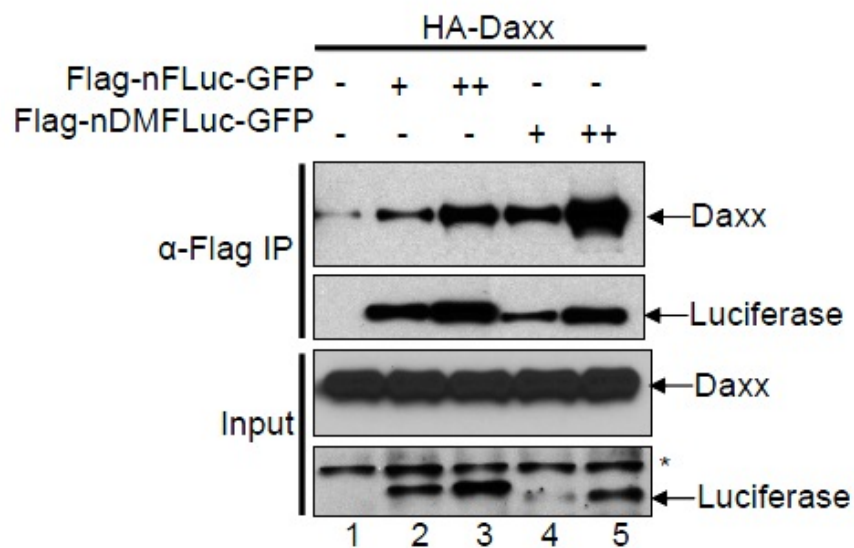
Figure 3.14. Daxx preferentially associates with an aggregation-prone luciferase mutant.
H1299 cells were transfected with HA-Daxx with Flag-nFluc-GFP or Flag-nDMFluc-GFP, an aggregation-prone mutant of luciferase, as indicated. Cell lysates were incubated with anti-Flag M2 beads and input and IP were analyzed by western blot. Asterisk indicates non-specific band.
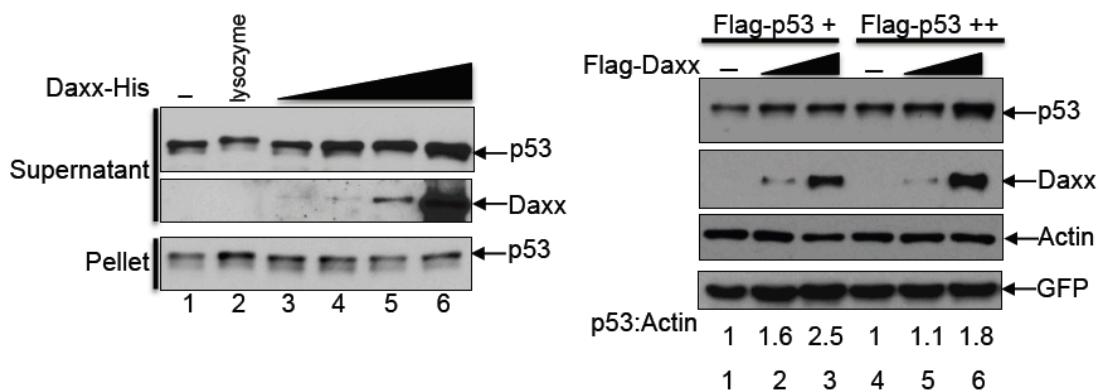
Figure 3.15. Daxx can renature p53 *in vitro* and *in vivo*.
(A) Heat-denatured p53 aggregates (35 nM) were incubated at 25°C for 3 h with lysozyme (100 nM), or Daxx (12.5 nM-100 nM). Reactions were then partitioned into soluble and insoluble fractions via sedimentation at 17,000 x g for 15 min. (B) *p53 -/-Mdm2 -/-* MEFs were transfected with increasing amounts of Flag-Daxx and two different amounts of Flag-p53. Samples were analyzed via SDS-PAGE and western blot.

states of Flag-p53, it was able to increase the protein levels of p53 (Figure 3.15B, lanes 2-3 vs. 1 and lanes 5-6 vs. 4). Together, these results suggest the Daxx is able to stabilize p53, when Mdm2 is not present, both *in vitro* and *in vivo*.

Overall, these results indicate that Daxx has the ability to interact with misfolded proteins and can assist in the renaturation of denatured substrates. This newly discovered function for Daxx might have great implications in terms of finding an explanation as to the many demonstrated functions of Daxx. It is possible that Daxx interacts with so many proteins in order to regulate their folding. In-depth discussion of these results can be found in chapter five.

MATERIALS AND METHODS

Antibodies, plasmids, and proteins – Antibodies for the following proteins/epitopes were purchased from the indicated sources: actin (A2066) and Flag (mouse monoclonal, M2, free (F3165) and conjugated to beads (A2220), HRP-conjugated (A8592), rabbit polyclonal (F7425)) (Sigma); GFP (JL-8, Clontech); Hausp/USP7 (A300, Bethyl Laboratories, Inc.); HA conjugated to HRP (Roche); luciferase (OB09, Calbiochem); Daxx (04-445, Millipore); p53 conjugated to HRP (DO-1, Santa Cruz Biotechnology). Plasmids encoding HA- or Flag-tagged Daxx and luciferase for transient transfection were made in pRK5. The luciferase plasmid was modified via addition of the SV40 nuclear localization signal (NLS) PKKKRKV and a Leu550-to-Ile mutation that abolishes the lysosomal localization of luciferase. Flag-nFluc-GFP and Flag-nDMFluc-GFP were modified from plasmids generously provided by Dr. S. Rayachaudhari (Gupta, Kasturi, et al, 2011). GFP-Hsp70 was constructed in the pEGFP-C3 vector. pGEX-GST-Daxx-His was constructed using a pGEX vector modified with a Tobacco Etch Virus cleavage site (ENLYFQ) between the GST tag and the N-terminus of Daxx and using PCR for Daxx and the 6xHis tag. All Daxx mutants were constructed similarly into the same modified pGEX vector. DaxxΔΔ was constructed using the QuikChange Site Directed Mutagenesis Kit (Agilent). Hsp104 was a kind gift from the lab of Dr. James Shorter. Hsp 70 and 40 were purchased from Enzo Life Sciences.

Protein purification – The pGEX-GST-Daxx-His vector was transformed into Rosetta 2 (Novagen) competent cells and resulting transformants were grown in 20 L LB medium, supplemented with 100 µg/mL ampicillin, for 14-16 h until they reached an OD_{600} of 0.5. Cultures were induced with 0.1-0.4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 27ºC. Cells were collected via centrifugation and the pellet was lysed with Ni-NTA

lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole at pH 8.0 containing fresh 1 mM PMSF, 2 mM DTT, and 1 mg/mL lysozyme) followed by sonication for 20 min. Lysates were either stored at -80ºC until needed or immediately incubated with GST beads (GE Healthcare) for 4 h to overnight at 4ºC. Beads were then washed with IP lysis buffer (20 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.5% NP-40, and 10% glycerol) twice, IP lysis buffer containing 0.5 M KCl once, IP lysis buffer containing 1.0 M KCl once, IP lysis buffer twice and AcTEV (Invitrogen) buffer (50 mM Tris-HCl at pH 8.0 and 0.5 mM EDTA) once. The beads were then incubated at 25ºC for 2-3 h with AcTEV protease (Invitrogen) in AcTEV buffer supplemented with 25 mM DTT. The supernatant was collected and incubated with Ni-NTA beads (Invitrogen) for 2-4 h at 4ºC. The beads were then washed with Ni-NTA wash buffer (50 mM NaH_2PO_4, 10 mM NaCl, and 10 mM imidazole at pH 7.0) three times and eluted with Ni-NTA elution buffer (50 mM NaH_2PO_4, 10 mM NaCl, and 500 mM imidazole at pH 7.0) for 1 h at 4ºC. The eluate was collected and subjected to IEX chromatography using a Mono Q column (GE) developed at a flow rate of 1 mL/min at pH 7.0. The protein was eluted from the column using a linear gradient of 10 mM – 1 M NaCl. Fractions were flash-frozen and stored at -80ºC until needed. Proteins were purified to apparent homogeneity as determined by SDS-PAGE and Coomassie staining and the identity of specific bands was confirmed via mass spectrometry analysis. When GST-tag cleavage was not desired, GST beads were eluted with 35 mM reduced glutathione (GSH; Sigma) at pH 7.5 for 1 h at 4ºC and the rest of the purification was the same as above. Flag-tagged p53 was purified from 293T cells using (10) 10 cm plates. Cells were transfected with via calcium phosphate transfection method. 16 h later, cells were harvested using IP lysis buffer. Lysates were spun down at 17,000 x g for 15 min at 4ºC and supernatants were incubated with anti-

Flag mAb beads for 4 h or overnight at 4ºC. After extensive washing, Flag-p53 was eluted using 3x Flag peptide (Sigma) for 1 h at 4ºC.

Luciferase assays – For renaturation assays, firefly luciferase (Sigma) was heat denatured at 42ºC for 10-15 min and distributed to reactions at a final concentration of 5 nM in luciferase refolding buffer (LRB: 25 mM HEPES-KOH at pH 7.4, 150 mM KAOc, 10 mM MgAOc, and 10 mM DTT). For these reactions, GST and lysozyme were used as negative controls, Hsp104/70/40 was supplemented with ATP and ATP regeneration solution (Boston Biochem) and used as a positive control, and Daxx and mutants were added without ATP. For prevention of denaturation assays, luciferase (5 nM) was concurrently incubated with proteins at 42ºC and readings were taken at specified times. In both cases, triplicate reactions were made and luciferase readings were taken using the Luciferase Assay System (Promega) at indicated times using a Monolight 2010 luminometer (Analytic Luminescence Laboratory). Activity was converted to fold change over native control via comparison to known quantities of an undenatured luciferase control.

Sedimentation assays – Luciferase and p53 were denatured at 42ºC or 37ºC for 15 min or 5 min, respectively, and then distributed to reactions containing buffer (LRB for luciferase; PBS with 5 mM DTT for p53), lysozyme as a negative control, or Daxx. After 3 h, the reactions were centrifuged at 17,000 x g for 15 min at 4ºC. The pellet and supernatant fractions were then resuspended in sample buffer and analyzed by SDS-PAGE.

Gel filtration – Luciferase (100 µM) was unfolded in 4 M Urea for 25 min at 30ºC and diluted 50-fold into cold LRB. Aggregates were then spun down at 17,000 x g for 10 min

at 4ºC. Then, 0.5 mL was loaded onto a 1 x 30 cm Superose 6 column (GE) equilibrated

in LRB. Fractions (0.4 mL) were collected 15 min post-injection at a flowrate of 0.4

mL/min. For heat-denatured luciferase, luciferase (1 µM) was incubated at 42ºC for 20

min or until the luciferase activity was at least two magnitudes less than native control.

Fractions were collected as for urea-denatured luciferase. For refolding in both

instances, column fractions in the range of 2000 kDa-44 kDa were incubated with LRB,

lysozyme (100 nM), Daxx (100 nM), or Hsp104/70/40 (100 nM) with ATP (10 mM) and

incubated at 25°C. Luciferase activity was determined after 90 min of refolding.

In vivo luciferase assay – H1299 cells were transfected with Flag-NLS-luciferase and

either Flag-pRK5, Flag-Daxx, or GFP-Hsp70. After 16 h, cells were split in triplicate into

24-well plates. 16 h later, cycloheximide (CHX) (50 µg/mL) was added to the cells for 30

min. The heat shock and recovery plates were placed at 45ºC for 30 min after which the

recovery plate was placed back at 37ºC for 3 h. Cells were lysed using Passive Lysis

Buffer (Promega) and read using the Luciferase Assay System (Promega). Readings

were normalized to the control sample of each group.

Immunoprecipitation and western blot – Transfections were carried out using

Lipofectamine 2000 (for DNA) or RNAiMAX (for siRNA) (Invitrogen) according to the

manufacturer's instructions. After treatments were complete, cells were lysed in IP lysis

buffer, supernatant (50 mM Tris at pH 8.8, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40, and

fresh 2 mM DTT, 250 IU/ml benzonase (Sigma), 1 mM PMSF, and 1x complete protease

inhibitor cocktail) or pellet buffer (20 mM Tris at pH 8.0, 15 mM $MgCl_2$, and fresh 2 mM

DTT, 250 IU/ml benzonase, 1 mM PMSF, and 1x complete protease inhibitor cocktail)

for fractionation analysis. Flag-NLS-luciferase was immunoprecipitated with anti-Flag

mAb beads and analyzed by western blot using anti-Luc antibody (1:500) for luciferase

input, Flag-HRP antibody (1:1000) for luciferase IP, and HA-HRP antibody (1:5000) for Daxx input and IP.

In vitro *binding* - Flag-NLS-luciferase was purified from 293T cells using anti-Flag mAb beads for 4 h to overnight at 4ºC and eluted with 3x Flag peptide for 1 h at 4ºC. GST, GST-Daxx, -D/E, and -PAH2 were constructed into pGEX with only a GST tag and purified similarly to pGEX-GST-Daxx-His proteins with omission of the His purification and IEX chromatography steps. Briefly, cleared lysates were incubated with GST beads for 4 h to overnight at 4ºC. After extensive washing, the beads were eluted with 30mM GSH at 4ºC for 1 h. Mixtures of luciferase (5 nM) and Daxx fragments were incubated at 4ºC for 2 h after which a portion was taken for input. The rest of the reactions were incubated with GST beads previously blocked with 3% BSA for 1 h at 4ºC. Samples were washed three times with IP lysis buffer and beads were resuspended in sample buffer and analyzed by SDS-PAGE and western blot. Input and IP samples were blotted with luciferase antibody and anti-GST antibody for GST, Daxx and Daxx mutants.

Quantitative RT-PCR analysis – Total RNA was isolated from cells using TRIzol (Invitrogen). Reverse transcription was performed using the First Strand cDNA Synthesis Kit (Marligen Biosciences). Primers used for luciferase amplification are as follows: forward primer 5' – CAA CTG CAT AAG GCT ATG AAG AGA – 3'; reverse primer 5' – ATT TGT ATT CAG CCC ATA TCG TTT – 3'.

CHAPTER 4: THE ROLE OF DAXX IN CDK6 REGULATION

<u>INTRODUCTION</u>

Daxx has the ability to regulate cellular proliferation through its cell-cycle dependent expression patterns and functions. Daxx is translocated to heterochromatin during S phase of the cell cycle via association with ATRX. It is released from ATRX during G2 phase, suggesting that the Daxx-ATRX interaction is important for S phase progression. Furthermore, Daxx-deficient cells display an accelerated S phase (Ishov, Vladimirova, and Maul, 2004). Another study uncovered a role for Daxx in cell cycle progression where depletion of Daxx led to perturbations in the duration of mitotic stages (Giovinazzi, Lindsay, et al, 2012). They found that Daxx-depleted cells displayed a stabilization of cyclin B and though there was a faster progression through prophase, there was a prolongation of prometaphase/metaphase, indicating that Daxx is required for normal mitosis. Preliminary data from our lab indicate that Daxx knockdown abrogates cell growth in a p53-independent and -dependent manner (Figure 4.1, A and B). When Daxx is stably knocked down using shRNA in U2OS or the p53-null cell line H1299, there is an abrogation of proliferation. Decrease in cellular proliferation in the p53 wild-type cells can be explained by the role of Daxx in Mdm2 stabilization (Tang, Qu, et al, 2006); however, the mechanism by which Daxx can regulate proliferation independent of p53 remains elusive. While investigating targets of Daxx using microarray analysis in human colon carcinoma (HCT116 p53 +/+) cells, we found that in Daxx-depleted cells, a cell cycle regulator, cyclin-dependent kinase 6 (Cdk6), was the most strongly downregulated gene (Figure 4.2).

Cdk6 was first identified as a novel member of the vertebrate family of Cdk1 kinases (Meyerson, Enders, et al, 1992). It was found to possess kinase activity toward

A
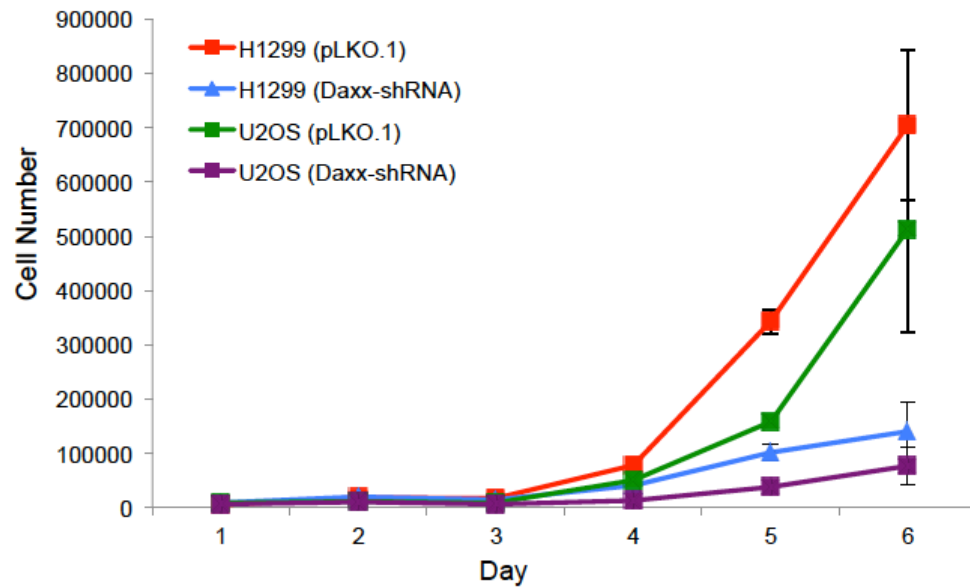
B
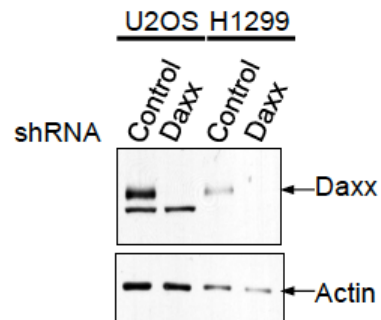
Figure 4.1. Daxx-depletion leads to abrogation of cell growth in a p53-independent manner.
(A, B) U2OS and H1299 (p53-deficient) cells were used to create stable cell lines expressing pLKO.1 or Daxx-shRNA and assessed for proliferation ability (n = 2) (A) and protein expression (B).
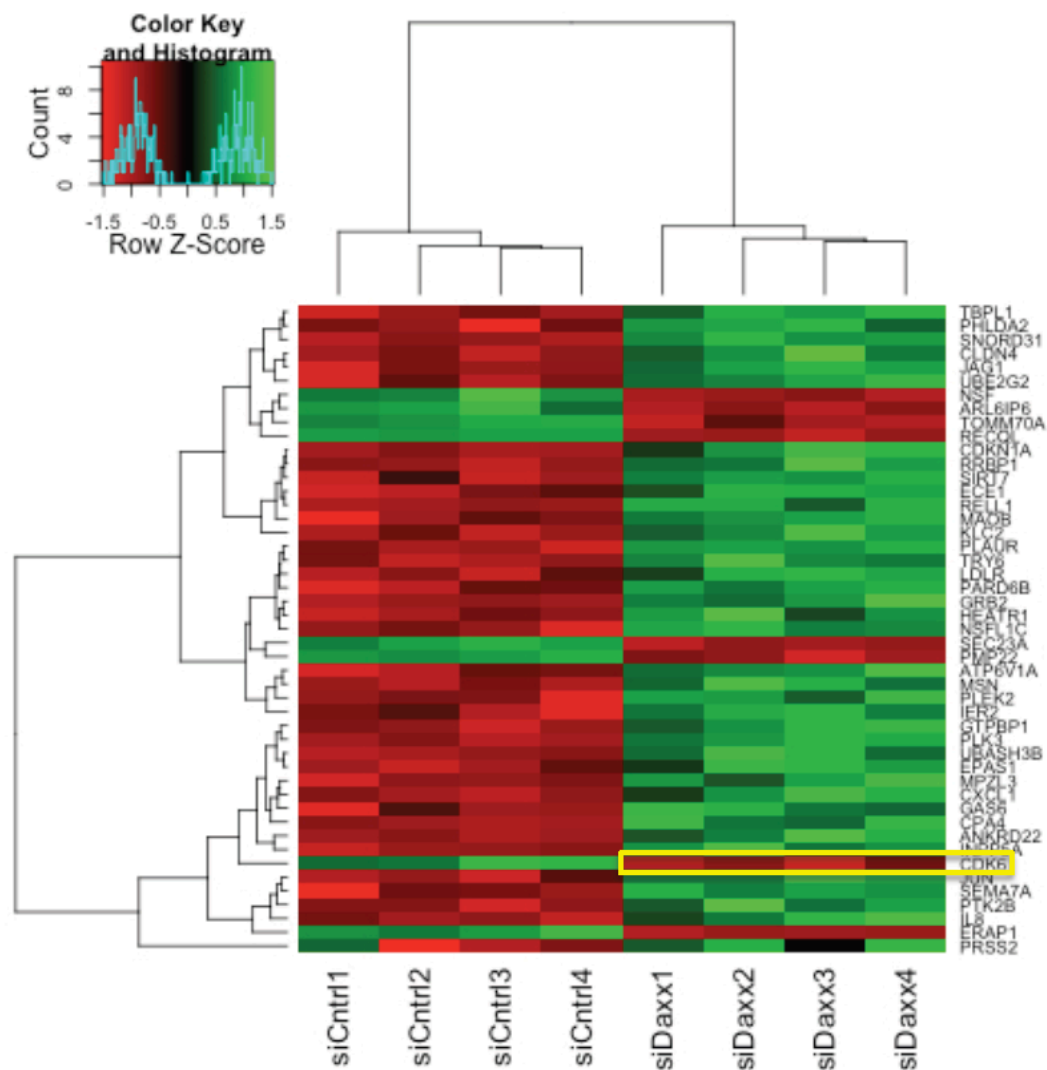
Figure 4.2. Heat map depicting microarray analysis of Daxx-depleted HCT116 p53 +/+ cells.
Yellow box highlights downregulation of Cdk6 expression upon Daxx silencing. Figure courtesy of Aaron Stonestrom.

pRb and partner with the G1 phase cyclins, allowing for progression through the cell cycle (Meyerson, and Harlow, 1994). Cdk6 shares almost 71% homology with Cdk4 and both are able to bind to cyclins D1, D2, and D3 during G1 phase to phosphorylate pRb (Grossel, and Hinds, 2006). Both are ubiquitously expressed and are generally considered functionally redundant (Grossel, and Hinds, 2006). However, recent evidence indicates that Cdk6 has functions distinct from those of Cdk4 and can have a role beyond cellular proliferation.

Many studies have emerged implicating Cdk6 as a regulator of differentiation in a variety of cell types. For example, Cdk6 can block differentiation of murine erythroleukemia (MEL) cells and its downregulation is required for MEL cells to reinitiate differentiation (Matushansky, Radparvar, and Skoultchi, 2003). The entry of MEL cells into terminal differentiation is accompanied by a decrease in Cdk2 followed by a decline in Cdk6 activity and inhibitors against Cdk2 and Cdk6 can trigger differentiation. Importantly, inhibitors against Cdk4 or an inhibitor-resistant form of Cdk4 cannot cause differentiation of these cell types, providing a functional difference between Cdk6 and Cdk4 (Matushansky, Radparvar, and Skoultchi, 2000). Further, it was shown that Cdk6 downregulation is essential for BMP-2-mediated osteoblast differentiation (Ogasawara, Kawaguchi, et al, 2004). Overexpression of Cdk6, but not Cdk4, led to inhibition of differentiation of the osteoblasts. Interestingly, this study found that Cdk6 did not influence the proliferation of the osteoblasts and had no effect on their progression through the cell cycle. Finally, Cdk6 is essential for Notch-dependent T-lineage survival, proliferation, and differentiation (Hu, Deshpande, et al, 2009). Cdk6, though ubiquitously expressed, is most abundant in lymphoid organs (Meyerson, Enders, et al, 1992; Meyerson, and Harlow, 1994). It has predominant expression in hematopoietic cell types

and is overexpressed in human T-cell lymphoblastic leukemia (T-ALL), suggesting a role for Cdk6 in thymocyte malignancies (Chilosi, Doglioni, et al, 1998; Della Ragione, Borriello, et al, 1997). The study by Hu, et al found that *Cdk6 -/-* mice had a reduction in thymocyte numbers in all compartments, consistent with the observation that *Cdk6 -/-* mice have defective thymic development (Malumbres, Sotillo, et al, 2004). It was found that Cdk6 is required for Notch-dependent proliferation, survival and differentiation. Together, these results suggest that Cdk6 has cell cycle-independent functions.

While Cdk6 is presumed to be a promoter of cellular proliferation, Cdk6 can also inhibit proliferation in a specific subset of cells. NIH 3T3 cells that stably overexpress Cdk6 proliferated at a significantly slower rate than when the cells concurrently overexpressed Cdk6 and a dominant negative form of Cdk6, indicating that Cdk6 was inhibiting cell growth (Nagasawa, Gelfand, and Lucas, 2001). Additionally, Cdk6 can inhibit the proliferation of some human mammary epithelial (HMEC) cells (Lucas, Domenico, and Gelfand, 2004). This study showed that breast-tumor derived cell lines had reduced levels of Cdk6 protein while primary HMECs had high levels of Cdk6. Overexpression of Cdk6 in the tumor cell lines led to reduced growth rates; when ectopic expression of Cdk6 was lost, they were able to proliferate more rapidly.

The roles of Cdk6 in differentiation and anti-proliferation indicate that Cdk6 is more than a mediator of the cell cycle. Understanding the regulation of Cdk6 would provide new insight into how cellular differentiation occurs and how cancer cells downregulate the growth-inhibitory effects of Cdk6 to promote proliferation. Based upon our microarray analysis showing that Cdk6 was strongly downregulated upon Daxx depletion and that Daxx may have p53-independent functions related to cellular proliferation, we focused our efforts on the regulation of Cdk6 by Daxx.

RESULTS

Daxx depletion leads to lower protein levels of Cdk6 in a variety of cell lines

To confirm the microarray result where Daxx depletion in HCT116 p53 +/+ cells led to downregulation of *Cdk6* (Figure 4.2), an array of cancer cell lines were treated with control or Daxx siRNA and assessed for Cdk6 expression. When Daxx was depleted in U2OS or isogenic HCT116 p53 +/+ and p53 -/- cells, Cdk6 protein levels were also dramatically reduced (Figure 4.3, A and B). This effect was also seen when the primary cell lines, IMR90 (human fetal lung fibroblasts) and MEFs (mouse embryonic fibroblasts) were used for analysis (Figure 4.3, C and D). Conversely, overexpression of Daxx in HCT116 p53 +/+ cells correlated with higher Cdk6 protein levels, albeit it was not a very robust effect (Figure 4.4A). A rescue experiment was performed using HCT116 p53 +/+ cells where Daxx was stably knocked down using shRNA. After establishing the stable cell line, Flag-Daxx was transiently overexpressed and Cdk6 levels were assessed. Upon expression of exogenous Daxx, Cdk6 levels were higher than when Daxx was depleted indicating that Daxx can modulate Cdk6 protein levels (Figure 4.4B, lanes 2-4 vs. 1).

To determine if the effect of Daxx was specific to Cdk6, we examined the expression of various cell cycle markers upon Daxx depletion. We examined the levels of Cdk2, Cdk4, cyclin D1, and Rb (Figure 4.5, A and B) in a variety of cancer cell lines. These proteins were minimally affected by Daxx knockdown suggesting that the effect of Daxx was specific for Cdk6.

Daxx affects transcription of *Cdk6*

To test if Daxx was regulating the stability of Cdk6 via a degradation-dependent
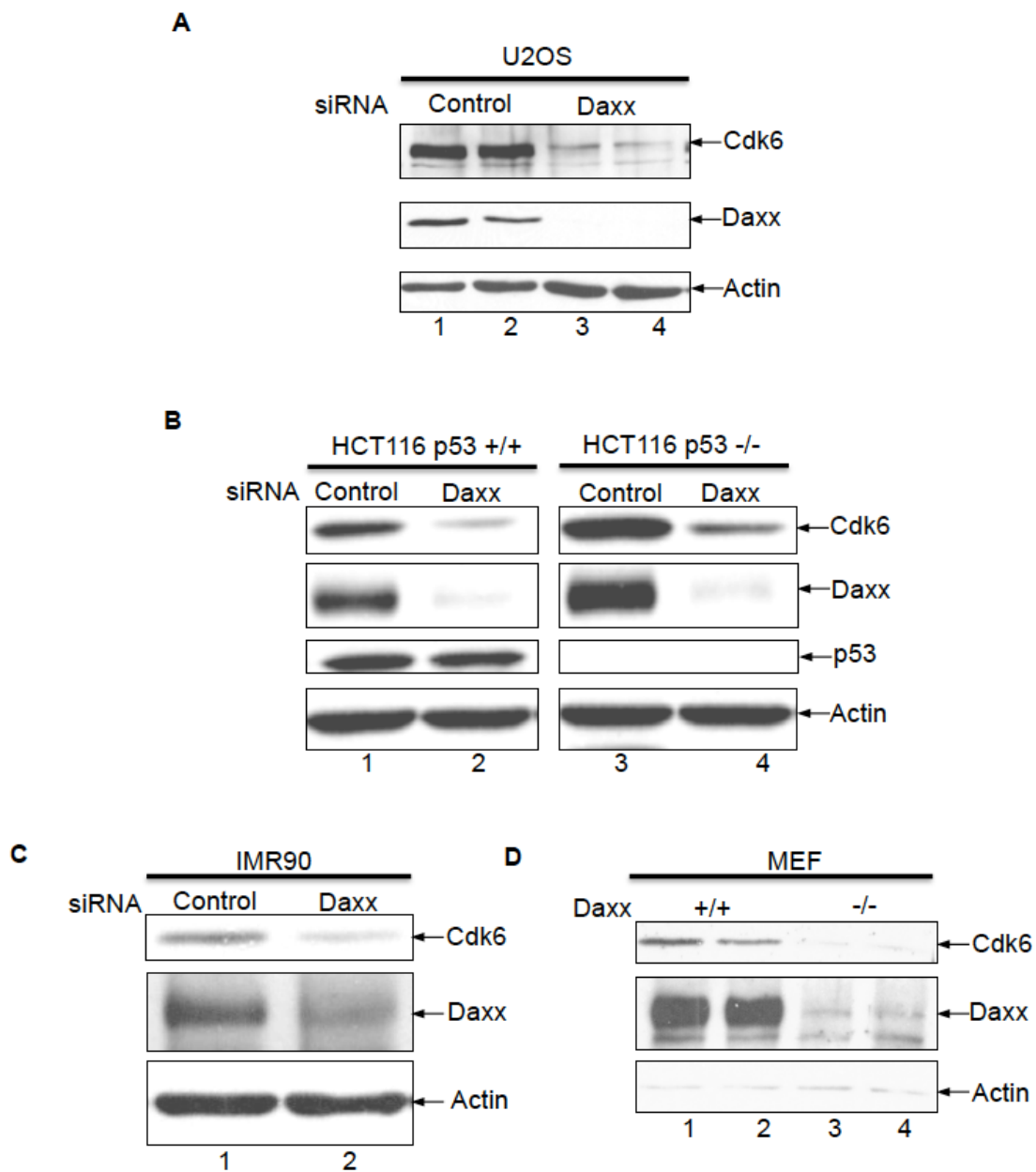
Figure 4.3. Cdk6 protein levels decrease upon Daxx silencing.
U2OS (A) HCT116 p53 +/+ and -/- (B) and IMR90 (C) cells were treated with control or
Daxx siRNA and analyzed for Cdk6 expression via western blot. (D) *Daxx +/+* or *Daxx -/-*
MEFs were assessed for Cdk6 expression via western blot.
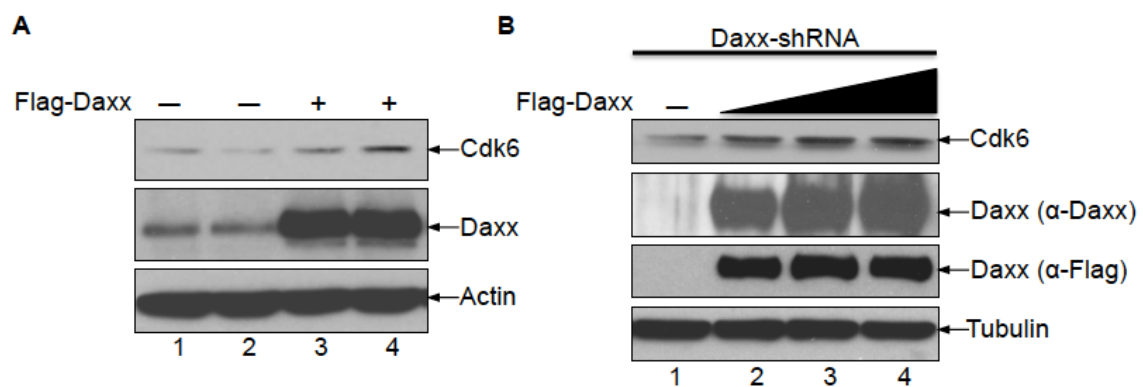
Figure 4.4. Daxx overexpression increases Cdk6 protein levels.
(A) Flag-Daxx was transfected in HCT116 +/+ cells and analyzed by western blot for Cdk6. Daxx levels were assessed using anti-Daxx antibody. (B) HCT116 p53 +/+ cells stably expressing Daxx shRNA were transfected with increasing amounts of Flag-Daxx and assessed for Cdk6 levels. Daxx expression was confirmed separately by anti-Daxx and anti-Flag antibodies.
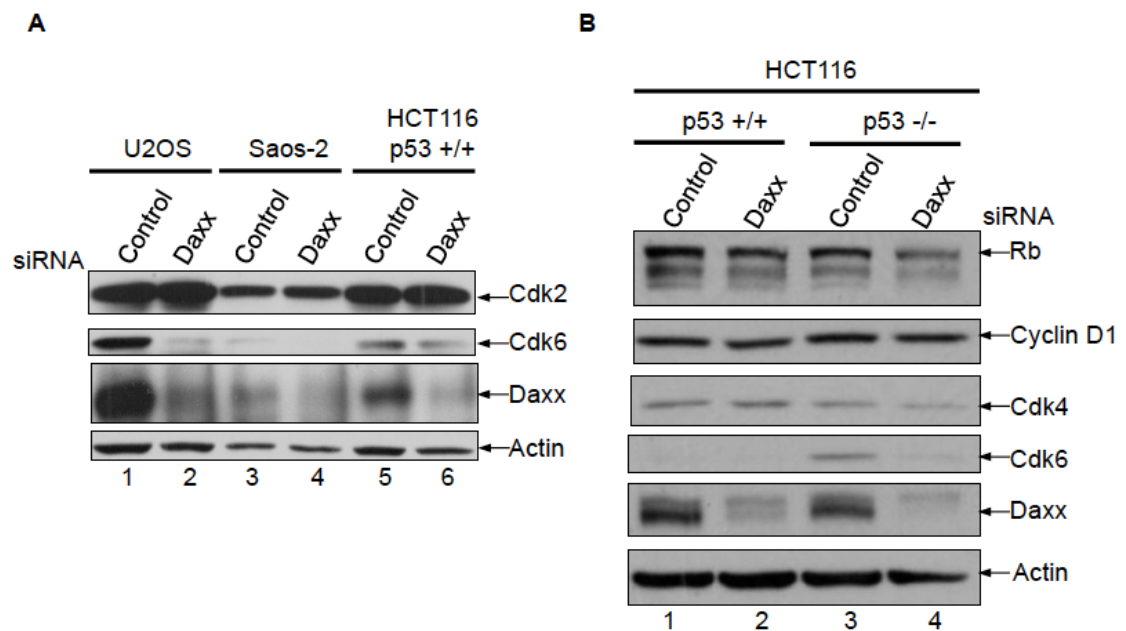
Figure 4.5. Daxx does not affect protein levels of other cell cycle regulators.
(A) U2OS, Saos-2, and HCT116 p53 +/+ cells were treated with control or Daxx siRNA and analyzed by western blot for Cdk2 and Cdk6 expression. (B) Isogenic HCT116 p53 +/+ and p53 -/- cells were Daxx-depleted and assessed for Rb, cyclin D1, and Cdk4 expression.

mechanism, we used MG132, a proteasome inhibitor, in combination with Daxx siRNA and examined Cdk6 protein levels in various cancer cell lines. We analyzed Mdm2 levels as an internal control for the effectiveness of MG132 treatment, as Mdm2 is very sensitive to proteasome-mediated degradation. We observed that with MG132 treatment, Mdm2 levels increased indicating that MG132 was inhibiting the proteasome and in turn Mdm2 degradation (Figure 4.6). Daxx silencing led to lower Cdk6 levels, yet Cdk6 levels were only marginally altered in the presence of MG132, regardless of Daxx siRNA treatment. This is not completely unexpected, as Cdk levels remain fairly constant during the cell cycle (Sherr, 2000; Vermeulen, Van Bockstaele, and Berneman, 2003). This result suggests that Daxx is not regulating Cdk6 levels via a degradation-dependent mechanism.

To further test if Daxx was controlling the stability of Cdk6 protein, cycloheximide was used to inhibit new protein synthesis in HCT116 p53 -/- cells to determine changes in Cdk6 half-life in the absence of Daxx. When Daxx was silenced via siRNA treatment, there were lower overall levels of Cdk6. To fairly compare the half-life of Cdk6 upon Daxx knockdown, we analyzed two different exposures of Cdk6 where the zero hour time points appeared most equal (Figure 4.7, lanes 5-8 vs. 1-4). After comparison of the different exposures, it was clear that Daxx depletion had little effect on the half-life of Cdk6. Together, these results indicate that Daxx does not affect the protein stability of Cdk6.

To further elucidate the mechanism by which Daxx was affecting Cdk6 protein levels, we examined if Daxx affected *Cdk6* transcription. Human cervix adenocarcinoma (HeLa) and H1299 cells were treated with Daxx siRNA and analyzed via western blot, RT-PCR and quantitative RT-PCR (Figure 4.8, A-C). Daxx-depleted cells displayed
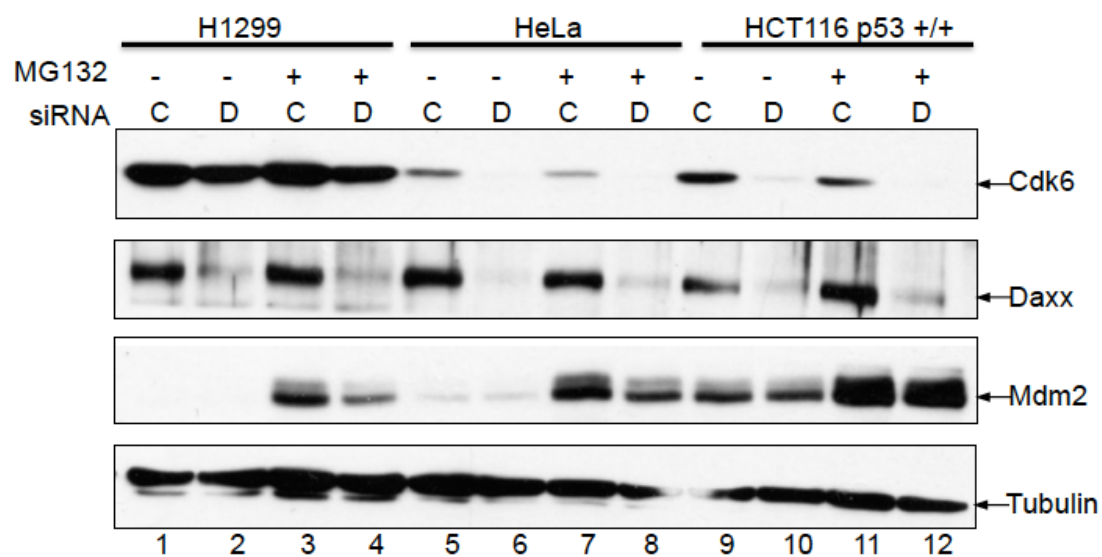
Figure 4.6. Daxx does not affect protein stability of Cdk6.
H1299, HeLa, and HCT116 p53 +/+ were treated with control or Daxx siRNA. 16 h later, cells were treated with MG132 (20 µM) for 4 h. Samples were harvested and analyzed via western blot.
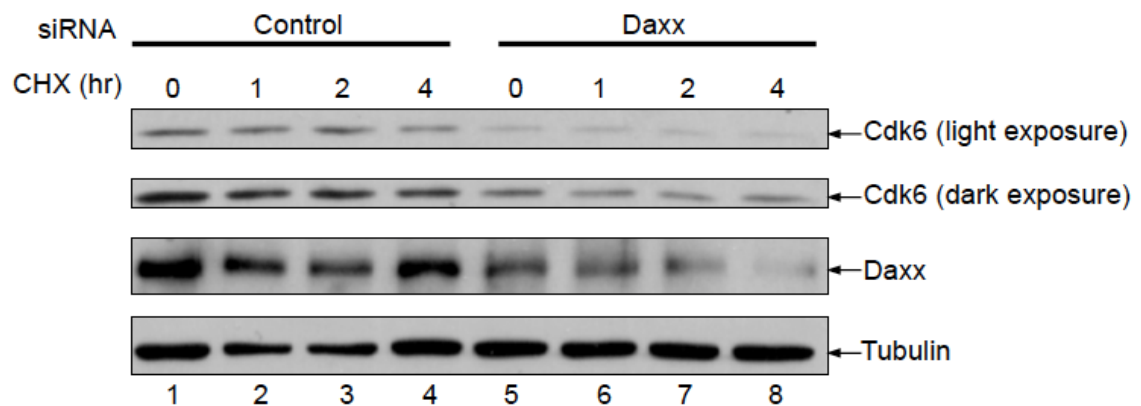
Figure 4.7. Daxx does not affect half-life of Cdk6.
HCT116 p53 -/- cells were depleted of Daxx using siRNA. 16 h later, cells were treated with CHX (50 μg/mL) for the indicated times. After harvesting and lysis, samples were analyzed for Cdk6 expression via western blot. Two different exposures of Cdk6 are shown where band intensity at the zero time points is most comparable.
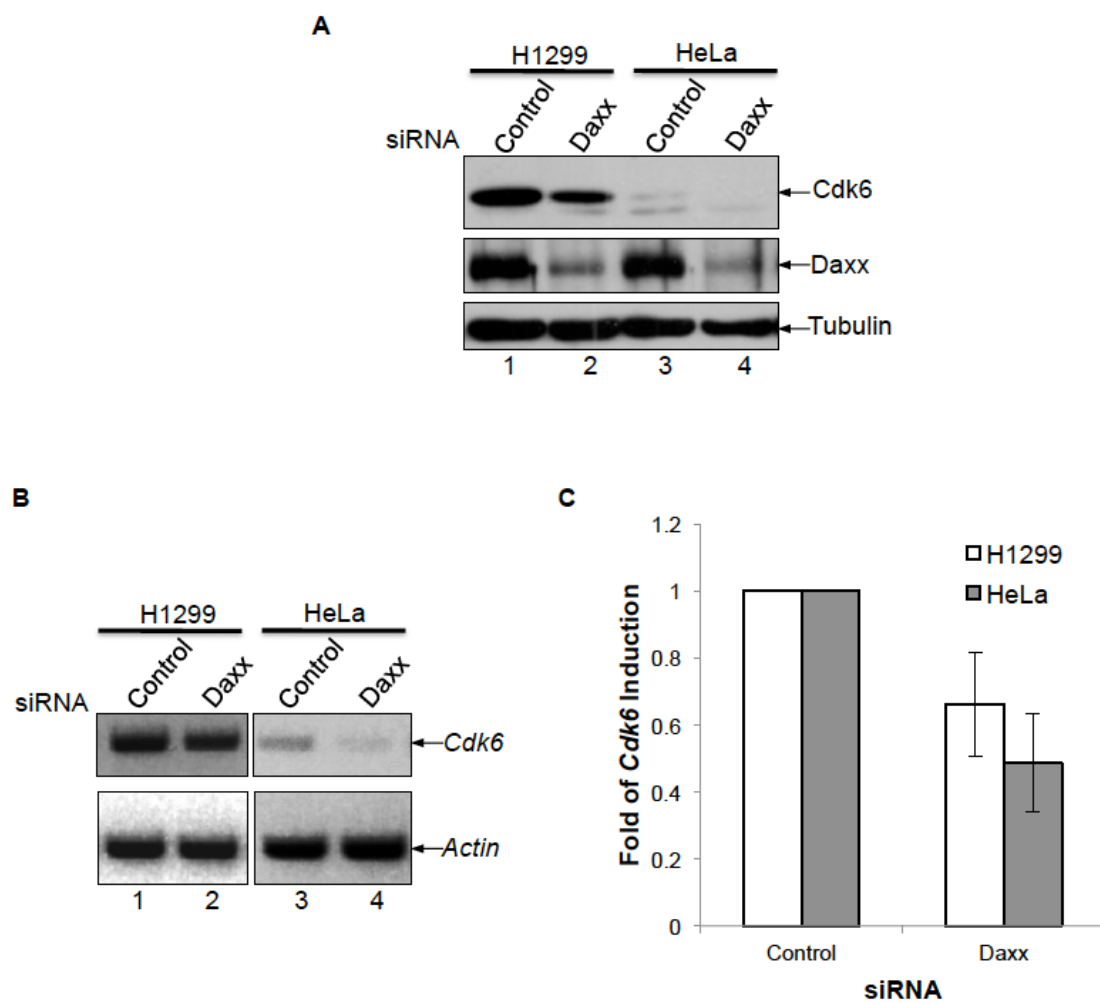
Figure 4.8. Daxx affects transcription of *Cdk6*.
H1299 and HeLa cells were treated with control or Daxx siRNA and assessed for protein (A) and mRNA levels using RT-PCR (B) and qPCR (C).

lower levels of Cdk6 protein (Figure 4.8A) and mRNA (Figure 4.8, B and C) suggesting

that Daxx was able to regulate the transcription of *Cdk6*. To expand the transcriptional

regulatory role of Daxx, we performed chromatin immunoprecipitation (ChIP) to assess

whether Daxx associates with the endogenous *Cdk6* promoter (Figure 4.9). For this

experiment, HCT116 p53 +/+ cells were subject to chemical crosslinking followed by

immunoprecipitation of Daxx and analysis of associated DNA via qPCR using gene-

specific primers (see materials and methods for sequences). The results are shown as

relative input units to account for background signal and input chromatin. The ChIP

assay reveals that Daxx bound to the Cdk6 promoter with some specificity, as it was not

associated with the *actin* promoter. p53 was immunoprecipitated as a positive control

and it bound to the p21 promoter, as expected. Daxx signal was most enriched at the -

700 bp region of the Cdk6 promoter. Interestingly, there is a potential binding NF-κB

binding site at this region of the *Cdk6* promoter (Cram, Liu, et al, 2001) and Daxx has

previously been shown to modulate transcription of NF-κB target genes (Michaelson,

and Leder, 2003; Park, Lee, et al, 2007). We also observed enrichment of Daxx at the -1

kb and +1 kb regions; it is possible that Daxx can bind in multiple areas of the *Cdk6*

promoter.

Daxx depletion does not affect the cell cycle

We next attempted to determine the consequence of Daxx knockdown and

subsequent decrease of Cdk6 levels on cellular proliferation by using HCT116 p53 +/+

and p53 -/- cells (Figure 4.10, A-C). Stable cell lines were created where Daxx was

silenced using shRNA and after establishment of the stable cell lines, proliferation
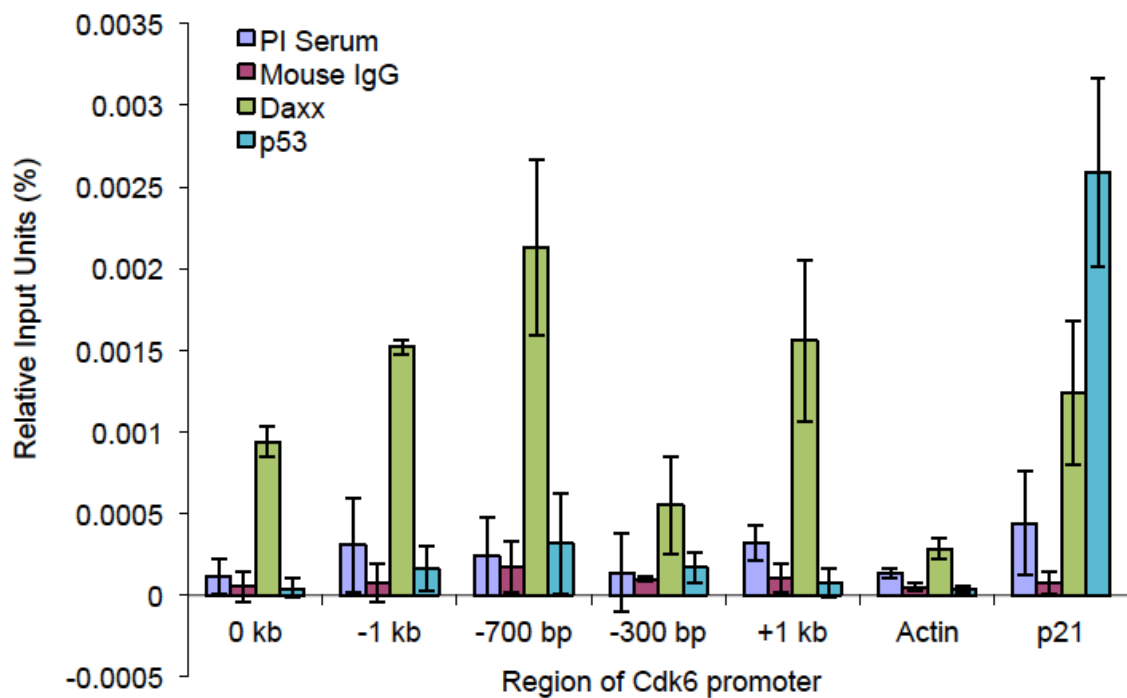
Figure 4.9. Daxx can bind to promoter of *Cdk6*.
Chromatin from HCT116 p53 +/+ cells was immunoprecipitated using anti-Daxx serum, anti-p53 antibody, and their respective controls. Different regions upstream or downstream of the *Cdk6* transcription start site were amplified using region-specific primers and analyzed via qPCR. Samples are represented as relative input units by comparing fold enrichment to signal amplification of input samples. PI – pre-immune serum.
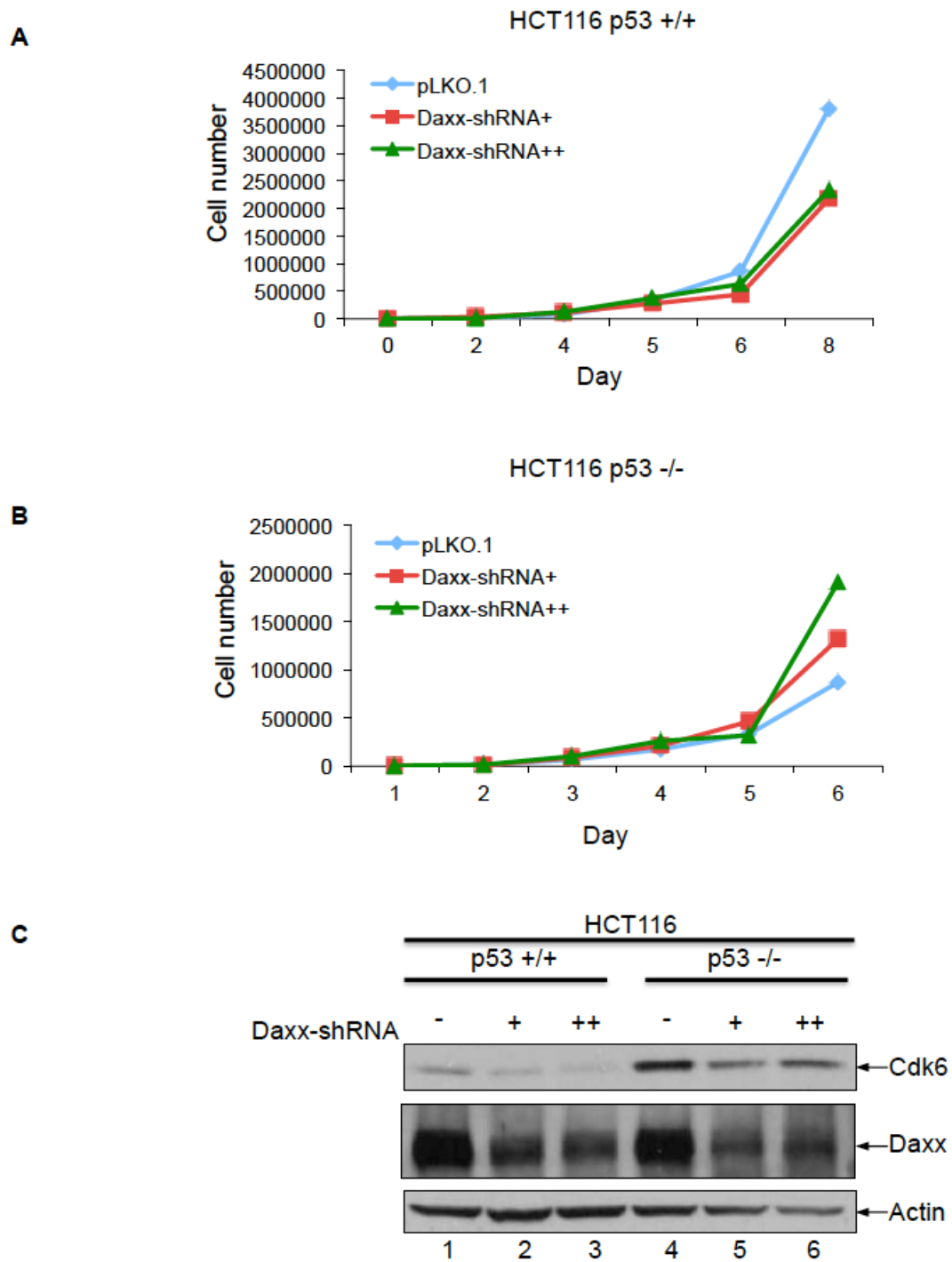
Figure 4.10. Daxx depletion and analysis of cellular proliferation.
(A-C) Stable cells lines expressing Daxx shRNA were established in HCT116 p53 +/+
and HCT116 p53 -/- cells. Cell lines were analyzed for proliferation (A and B) and protein
levels of Daxx and Cdk6 (C).

assays were performed. When Daxx was stably knocked down in HCT116 p53 +/+ cells, cellular proliferation occurred at a slower rate (Figure 4.10A); however, the same effect was not observed in HCT116 p53 -/- cells despite decreased levels of Cdk6 protein in the Daxx-depleted cells (Figure 4.10, B and C). Data from Figure 4.1 indicated that when Daxx was knocked down, there was a proliferative defect regardless of p53 status suggesting that this current result (Figure 4.10) may be cell type-specific. Based upon this observation, we sought to examine if Daxx depletion was able to alter the cell cycle profile. We used flow cytometry and propidium iodide (PI) staining to assess the cell cycle profiles of cells treated with control or Daxx siRNA. When Daxx was depleted in HCT116 p53+/+ or p53 -/- cells, we saw no difference in cell cycle profile compared to cells with control siRNA (Figure 4.11A). Daxx knockdown was confirmed via western (Figure 4.11B). These results suggest that while Daxx can affect the transcription of *Cdk6*, it is unable to elicit major changes in cellular proliferation or the duration of different stages of the cell cycle due to downregulation of Cdk6.

While we were able to confirm that Daxx depletion led to lower levels of Cdk6 at the protein and mRNA level and that Daxx is able to bind to the promoter of *Cdk6*, we were not able to establish a physiologically relevant outcome for this effect. Future studies will determine the mechanism and consequence of this newly identified function of Daxx. A discussion of the current results can be found in chapter five.
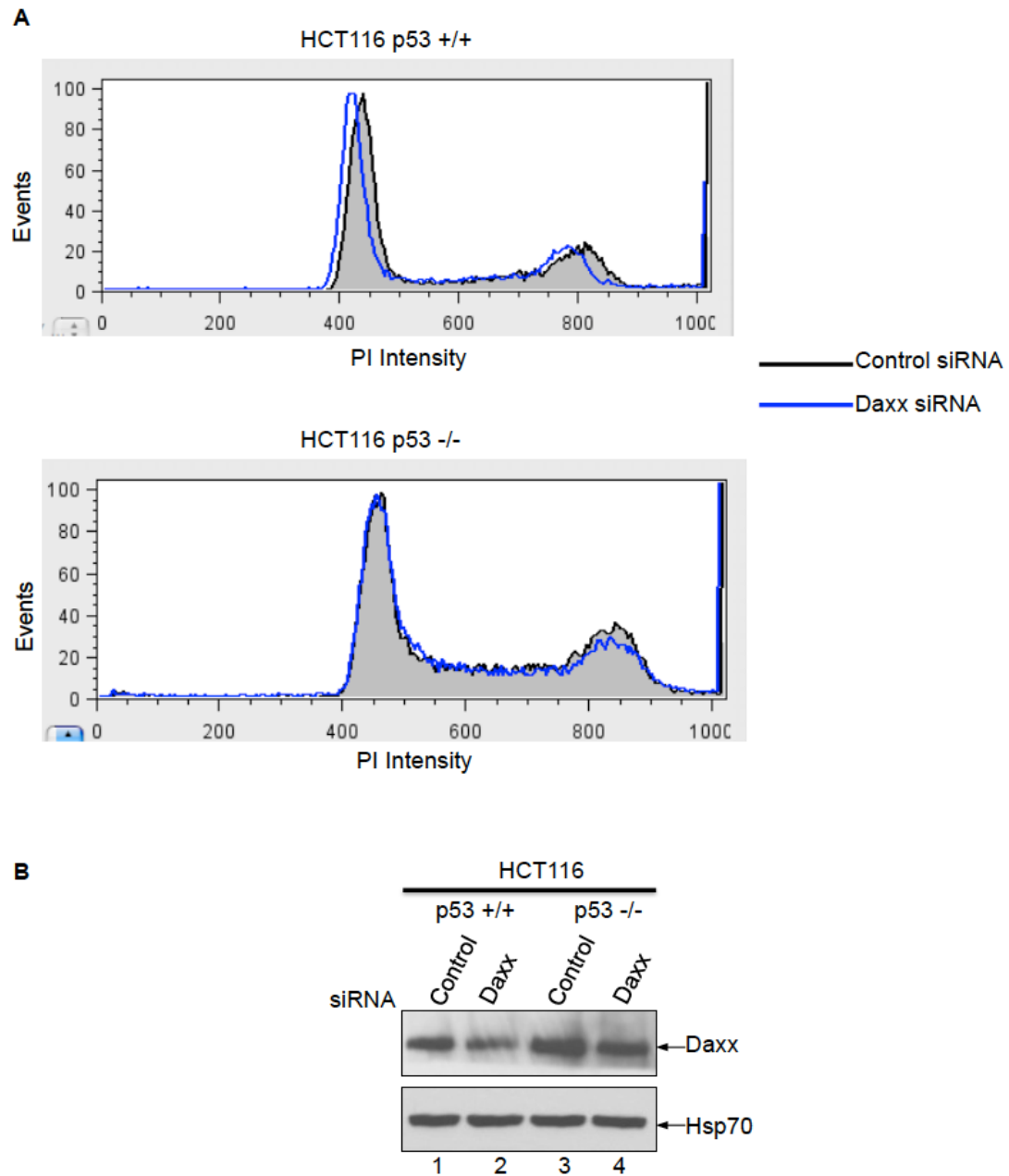
Figure 4.11. Daxx silencing does not affect cell cycle profile.
HCT116 p53 +/+ and HCT116 p53 -/- cells were treated with control and Daxx siRNA. 16 h later, cells were stained for PI and assessed for cell cycle distribution (A). Western analysis confirms Daxx knockdown. Hsp70 is shown as loading control (B).

MATERIALS AND METHODS

Antibodies - Antibodies for the following proteins/epitopes were purchased from the indicated sources: actin (A2066), tubulin (T4026), and Flag (mouse monoclonal, M2, (F3615) (Sigma); Daxx (M-112) p53 (DO-1) (Santa Cruz Biotechnology); Cdk6 (DCS83) and Cyclin D1 (DCS6) (Cell Signaling); GFP (JL-8, Clontech); Mdm2 (Ab-1,Calbiochem); Hsp70 (C92F3A-5, Enzo Life Sciences). Antibodies for Cdk2 and Cdk4 were kind gifts from the lab of Dr. J. Alan Diehl. Antibody for Rb was a kind gift from the lab of Dr. Judy Meinkoth.

Microarray analysis – Note: Performed by Aaron Stonestrom. HCT116 p53 +/+ cells were transfected with control or Daxx siRNA and total RNA was isolated using TRIzol (Invitrogen). Samples were then sent to the Penn Molecular Profiling Facility for analysis. Briefly, total RNA was converted to first-strand cDNA using Superscript II reverse transcriptase primed by a poly(T) oligomer that incorporated the T7 promoter. Second-strand cDNA synthesis was followed by *in vitro* transcription for linear amplification of each transcript and incorporation of biotinylated CTP and UTP. The cRNA products were fragmented to 200 nucleotides or less, heated at 99ºC for 5 min and hybridized for 16 h at 45ºC to microarrays. The microarrays were then washed at low (6X SSPE) and high (100mM MES, 0.1M NaCl) stringency and stained with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycoerythrin stain. A confocal scanner was used to collect fluorescence signal at 3um resolution after excitation at 570 nm. The average signal from two sequential scans was calculated for each microarray feature. Affymetrix Microarray Suite 5.0 was used to quantitate expression levels for targeted genes; default values provided by Affymetrix were applied to all analysis parameters.

Quantitative RT-PCR analysis - Total RNA was isolated from cells using TRIzol (Invitrogen). Reverse transcription was performed using the First Strand cDNA Synthesis Kit (Marligen Biosciences). Primers used for Cdk6 amplification are as follows: forward primer 5' – GTA ATC GTG TCT GTG TTG AG – 3'; reverse primer 5' – TCT GCA CCC GCA CGC GCT TC – 3'.

Cell culture, transfections, and western blot - All cells were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. To generate lentiviruses expressing control and Daxx shRNA, which were cloned into the pLKO.1 backbone, phoenix cells were transfected with pREV, pGAG, and pVSVG with either control (pLKO.1) or Daxx shRNA. 48-72 h after transfection, the lentivirus-containing medium was used to infect HCT116 p53+/+ and p53 -/- cells in the presence of 8 µg/mL polybrene. The infected cells were selected in the presence of 2 µg/ml puromycin for 4-5 days. Transient transfections were carried out using Lipofectamine 2000 (for DNA) or RNAiMAX (for siRNA) (Invitrogen) according to the manufacturer's instructions. MG132 (20 µM) was applied to cells for 4 h, when required. To harvest cells, briefly, cells were scraped in IP lysis buffer and spun down at 17,000 x g for 15 min at 4ºC. After protein concentration was determined (Bio-Rad), samples were resolved by SDS-PAGE. Samples were blotted with anti-Daxx (1:5000), anti-Cdk6 (1:200), anti-tubulin (1:5000), and anti-actin (1:5000) antibodies.

Cellular proliferation assay – After stable cells lines expressing Daxx shRNA were selected and assessed for Daxx knockdown, 5,000 cells per well were seeded in 24-well, 12-well, and 6-well plates and 6 and 10 cm dishes. Cells were seeded in duplicate for each condition and counted in successively larger plates to prevent them from reaching confluency.

Cell cycle analysis – After Daxx silencing for 24 h, cells were collected via centrifugation and washed twice with PBS and then fixed with 70% ethanol overnight at −20 °C. Cells were then treated with 0.1% Triton X-100 and 50 μg/mL RNase A for 30 min at 37 °C followed by PI staining. Cell cycle distribution was analyzed using a FACScalibur flow cytometer (BD Biosciences). The data were analyzed using Flow Jo software (Tree Star).

Chromatin Immunoprecipitation (ChIP) – ChIP was performed using the ChIP-IT Express kit according to manufacturer's instructions (Active Motif). HCT116 p53 +/+ cells were washed with PBS and crosslinked with a 1% formaldehyde solution for 10 min at 25ºC. Crosslinking was quenched by the addition of 125 mM glycine. Cells were collected using the provided cell-scraping solution supplemented with fresh 1 mM PMSF. Lysates were sonicated to generate DNA fragments with an average size below 1,000 base pairs and followed by immunoprecipitation with anti-Daxx serum, pre-immune serum, anti-p53 (DO-1), an isotype-matching control and provided Protein G magnetic beads at 4ºC for at least 4 h or overnight. Chromatin was eluted using provided elution buffer for 15 min at 25ºC and crosslinks were reversed using reverse cross-linking buffer. Proteinase K was added to the samples and incubated at 37ºC for 1 h. qPCR was performed on the eluted chromatin. The primer pairs were: -1 kb forward: GCT GTG CAA TGT GTT TGT GTT, -1 kb reverse: ACC TCC CAG GCT CAA CCT AT; +1 kb forward: ATG GAG GGA TGG TTG CTA GA, +1 kb reverse: GCA CAG CTC TCT GAA AGC AA; 0 kb forward: CGG CCA ATT GTT GAA TGA GT, 0 kb reverse: TGG TGC TTC TTA TTT GCA ACCA; -700 bp forward: TAA GTG CCC TTT GCA GGT G, -700 bp reverse: TGG TGT AGC CTA AGC CTC CT; -300 bp forward: CTC CAC TTC CCA GGT TCA AG, -300 bp reverse: AAC GTG GTG AAA CCC TGT GT; p21 forward: CTG AAA ACA GGC AGC

CCA AG, p21 reverse: GTG GCT CTG ATT GGC TTT CTG.

CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

In the preceding chapters, I have demonstrated that Daxx is involved in p53 regulation, protein folding, and Cdk6 modulation; an array of diverse activities that further add to the complex functions of Daxx within the cell and may also provide a biochemical unity for these functions. These findings further our understanding of the anti-apoptotic and transcriptional functions of Daxx while providing a novel role for Daxx in protein folding. The remainder of this chapter discusses the implications of these findings and provides ideas to expand these observations.

The Role of Daxx in p53 Regulation

The accumulation of p53 in response to cellular stress is highly dependent upon the destabilization of its main negative regulator, Mdm2. The mechanism of Mdm2 inactivation upon stress signaling is not well understood. In this section, we showed that Daxx is phosphorylated by ATM at Ser564 in response to DNA damage. This phosphorylation event hinders Daxx association with Mdm2 allowing for destabilization of Mdm2 and subsequent p53 activation (Figure 5.1). The stabilization of Mdm2 by the Daxx S5645A mutant is Hausp-dependent, confirming our previous study where wild-type Daxx mediated stabilization of Mdm2 was Hausp-dependent (Tang, Qu, et al, 2006).

ATM is activated upon DNA damage and leads to the phosphorylation of various substrates either directly or via activation of downstream kinases. We have discovered Daxx as a novel substrate of ATM. The finding that ATM not only phosphorylates Mdm2 and p53 in times of cellular stress, but also their upstream regulator, Daxx, indicates that the cell possesses an intricate signaling network to maintain genomic stability by activating p53. It has previously been shown that ATM-mediated phosphorylation of
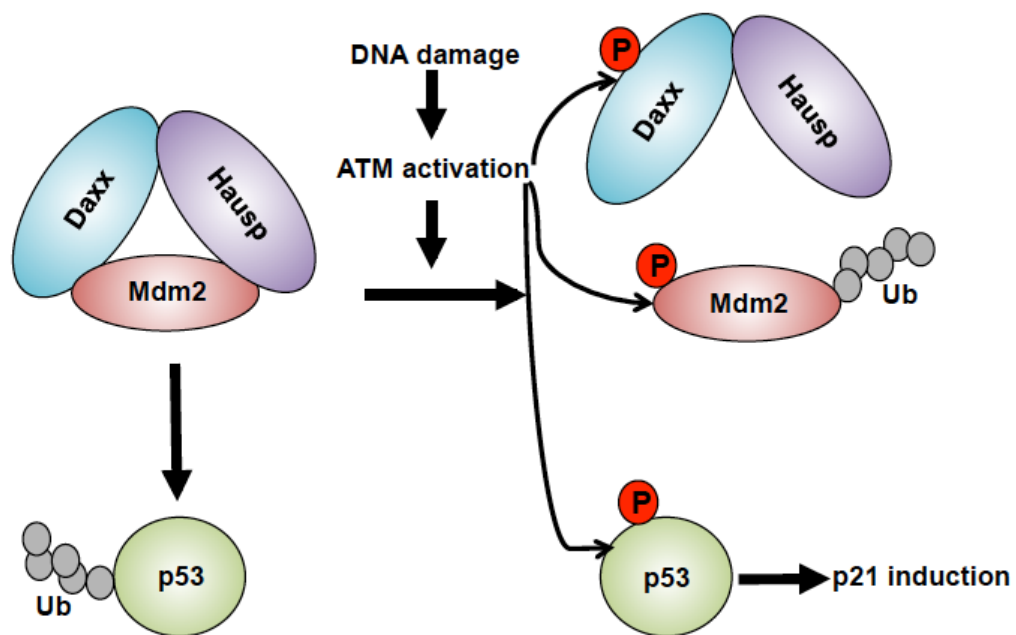
Figure 5.1. Phosphorylation of Daxx by ATM upon DNA damage allows for p53 activation.
Upon DNA damage, ATM phosphorylates Mdm2 and p53. Our finding extends its phosphorylation activity to Daxx, as well. This event hinders the association between Daxx and Mdm2 thereby disrupting the Daxx-Mdm2-Hausp complex, allowing for p53 activation.

Mdm2, and a related protein MdmX, reduces their affinity for Hausp, ultimately leading to p53 activation (Meulmeester, Maurice, et al, 2005). However, it is also known that Hausp deubiquitinates p53, which is important for p53 stabilization (Li, Chen, et al, 2002). These findings fit a model whereby the phosphorylation of Daxx upon DNA damage leads to dissociation of Mdm2 from itself and Hausp, allowing Hausp to direct its deubiquitinase activity toward p53 instead of Mdm2. Therefore, Daxx may provide Hausp with substrate specificity.

A major shortcoming of the present study was the lack of a strong phenotype upon the introduction of the Daxx S564A mutant. It is possible that the endogenous wild-type Daxx masked the effect of the mutant Daxx and that Mdm2 stabilization was already at a maximum. We attempted to circumvent this issue via the creation of stable cell lines that expressed shRNA against Daxx while also stably overexpressing either wild-type or mutant Daxx; however, we were unsuccessful in our attempts. It is possible that a Daxx-depleted background would allow for the effects of the mutant to become more apparent allowing us to see stronger downstream effects of p53 activation such as cell cycle arrest, apoptosis, or the induction of other p53 targets.

The discovery that Daxx is a novel target for ATM sheds further light on the many signaling pathways that govern p53 activation. Daxx appears to be the central component of the interaction between Mdm2 and Hausp, both of which are critical regulators of p53. Daxx may be a promising therapeutic target for tumors that retain wild-type p53, perhaps via the overexpression of Mdm2. The possibility cannot be excluded that the Daxx-Mdm2-Hausp complex not only regulates p53, but also other substrates of Mdm2. For example, the p53 homologs, p63 and p73 have been shown to interact with Mdm2 (Zdzalik, Pustelny, et al, 2010) with Mdm2 being able to inhibit p63 transcriptional activity and p63-mediated induction of apoptosis (Kadakia, Slader, and Berberich, 2001).

Thus, the stabilization of Mdm2 by Daxx not only has important implications for p53 regulation, but also for other potential substrates of Mdm2, many of which may have unexplored tumorigenic properties.

Future Directions

Another responder to DNA damage, besides ATM, is ATR (ataxia telangiectasia and Rad3-related protein). ATR mainly responds to single-stranded DNA, while ATM responds to double-strand breaks in DNA or chromatin disruption (Sancar, Lindsey-Boltz, et al, 2004). In some experiments, basal Daxx phosphorylation exists even without the presence of ATM or a DNA damaging agent. This can be explained in two ways: first, cancer cells may possess a basal amount of DNA damage leading to the activation of ATM causing phosphorylation of Daxx. Second, Daxx may be a substrate of ATR, as it is known that ATR phosphorylates p53 upon DNA damage suggesting that it may also target upstream regulators of p53 (Matsuoka, Ballif, et al, 2007; Tibbetts, Brumbaugh, et al, 1999). While ATM ablation in the study by Tang, et al did generally prevent the dissociation of the Daxx-Mdm2-Hausp complex upon DNA damage, it was not able to completely keep the complex from disrupting (Tang, Qu, et al, 2006). This indicates that another upstream regulator may be targeting the complex; a potential candidate is ATR. We could perform *in vitro* kinase assays with ATR to determine if ATR can directly phosphorylate Daxx. Since the consensus sites of ATM and ATR are the same, it is possible that ATM and ATR are acting at the same site to phosphorylate Daxx.

Besides examining the regulation of Daxx, we should also analyze how Daxx may affect other potential components of this complex. It is believed that Mdm2 exists predominantly as a heterodimer with a related protein, MdmX, and this heterodimerization increases the E3 activity of Mdm2 toward p53 (Kawai, Lopez-

Pajares, et al, 2007; Linares, Hengstermann, et al, 2003). MdmX is also able to prevent Mdm2 from degradation (Stad, Little, et al, 2001; Tanimura, Ohtsuka, et al, 1999). MdmX shares a few functional characteristics with Mdm2 in that it is overexpressed in many tumors that retain wild-type p53, it is able to inhibit the transactivation of p53, and *MdmX* -/- mice display embryonic lethality that can be rescued by simultaneous p53 deletion (Danovi, Meulmeester, et al, 2004; Finch, Donoviel, et al, 2002; Migliorini, Lazzerini Denchi, et al, 2002; Okamoto, Taya, and Nakagama, 2009). Due to their similarity in function, it would be interesting to see if Daxx is able to stabilize MdmX in the same way as it does for Mdm2. It would also be informative to examine if Daxx is able to interact with MdmX and whether this interaction is affected by DNA damage.

During the course of this study, a report discovered many additional sites of ATM-mediated phosphorylation on Mdm2 (Cheng, Chen, et al, 2009). We obtained Mdm2 mutants from this group that were either phosphorylation-resistant (Mdm2 6A) or phospho-mimics (Mdm2 6D). Preliminary data indicated that Daxx could enhance the stability of Mdm2 6A, but not Mdm2 6D. This is in agreement with our findings that phosphorylation is able to interrupt the interaction between Daxx and Mdm2. It would be informative to see if phosphorylation events on both Daxx and Mdm2 act in a synergistic manner to activate p53, as the effect of the Daxx mutant alone was quite modest. Additionally, the binding between Daxx and Hausp is interrupted upon DNA damage; however, use of the Daxx S564A mutant does not prevent the dissociation of Daxx and Hausp (unpublished results from Dr. Jun Tang), indicating that an unidentified phosphorylation event on Hausp may be regulating this interaction. Determining if this phosphorylation occurs due to ATM signaling would provide yet another mode of p53 regulation through the Daxx-Mdm2-Hausp complex.

Mdm2 activity cannot solely be controlled by Daxx, but also by the ARF tumor suppressor. ARF upregulation is often induced by the overexpression of oncogenes such as activated Ras and c-Myc (Groth, Weber, et al, 2000; Zindy, Eischen, et al, 1998). It is known that ARF can inhibit the E3 ubiquitin ligase activity of MDM2, resulting in p53-mediated apoptosis or cell cycle arrest (Kamijo, Weber, et al, 1998; Pomerantz, Schreiber-Agus, et al, 1998; Stott, Bates, et al, 1998). ARF can also interact with Daxx and induce its nucleolar translocation in addition to enhancing the SUMOylation and ubiquitination of Daxx, without affecting its protein levels (Ivanchuk, Mondal, and Rutka, 2008). Ivanchuk, et al showed that Daxx expression interferes with the ability of ARF to induce Mdm2 SUMOylation, thereby increasing the ubiquitination of Mdm2 and allowing for p53 activation; it is thought that inhibition of Mdm2 SUMOylation is required to upregulate Mdm2 self-ubiquitination (Lee, Lee, et al, 2006). The study by this group suggests that the interaction between ARF and Daxx influences the post-translational modifications of Mdm2 that are required to inhibit Mdm2-mediated p53 ubiquitination during cellular stress. It is possible that ARF is able to disrupt the Daxx-Mdm2-Hausp complex upon induction of stress signals via binding to Daxx and Mdm2 separately and mediating their post-translational modifications. There is a previously established ARF-inducible U2OS cell line that we could use to test how induction of ARF affects binding between components of the Daxx-Mdm2-Hausp complex. Moreover, it would be interesting to see if ARF is able to modify or interact with Hausp, thereby modulating the activity of Hausp toward Mdm2. Elucidating if ARF can affect the Daxx-Mdm2-Hausp complex would demonstrate that this complex is regulated by various cellular stresses using different mechanisms, denoting this complex as a central regulator of p53 activity.

Daxx as a Novel Molecular Chaperone

Daxx has been implicated in a variety of cellular functions since its discovery. This study uncovers yet another potential biological role of Daxx as a molecular chaperone, which may serve as a way to unify the diverse functions of Daxx. Our *in vitro* studies demonstrate that Daxx can refold misfolded substrates; however, it has minimal activity toward larger aggregates. Consistent with the *in vitro* results, we saw that Daxx could decrease the protein levels of insoluble luciferase and preferentially bound to heat-shocked luciferase. Additionally, Daxx showed refolding activity toward p53 both *in vitro* and *in vivo*.

Our results indicate that the PAH2 and acid-rich domains of Daxx are important for its activity. Although, it has previously been reported that the domains denoted as paired amphipathic helical domains are a misnomer (Escobar-Cabrera, Lau, et al, 2010), we still feel that the crude structural boundary we have used defines a domain important for the activity of Daxx. In addition, we found that the acid-rich region of Daxx imparts activity to misfolded luciferase. Both regions are well conserved through evolution in larger mammals (Figure 5.2). These observations support an evolutionarily conserved function for these two domains. The creation of point mutants or small deletion mutants within the conserved areas of the PAH2 and/or D/E domain would allow us to test exactly which residues of Daxx are interacting with misfolding species. In fact, it is known that the binding site for p53 lies within the D/E region of Daxx (Zhao, Liu, et al, 2004). It would be interesting to test if the aforementioned mutants would be able to solubilize misfolded p53. Moreover, we should assay if the DaxxΔΔ mutant or the PAH2 and D/E domains alone can act toward p53 renaturation.

A

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          151      ▼
Homo Sapiens    LAPAATTSNE PSGNNPPTHL SLDPTNAENT ASQSPRTRGS RRQIQRLEQL
Bos Taurus      LAPAATTSSE PSGNNPPTDP SSDP.NAETT ASEAPRTHGS RRQIQRLEQL
Mus musculus    LAPAASTTSE ASGPNPPTEP PSDLTNTENT ASEASRTRGS RRQIQRLEQL
Rattus norvegicus LAPAASA..E SSGDNPPTDP PSDLTNTETT ASEASRTRGS RRQIQRLEQL

          201                                              250
Homo Sapiens    LALYVAEIRR LQEKELDLSE LDDPDSAYLQ EARLKRKLIR LFGRLCELKD
Bos Taurus      LALYVAEIRR LQEKELDLSE MDDPDSTYLQ EARLKRKLIR LFGRLCELKD
Mus musculus    LALYVAEIRR LQEKELDLSE LDDPDSSYLQ EARLKRKLIR LFGRLCELKD
Rattus norvegicus LALYVAEIQR LQEKELDLSE LDDPDSSYLQ EARLKRKLIR LFGRLCDLKD

          251                                              300
Homo Sapiens    CSSLTGRVIE QRIPYRGTRY PEVNRRIERL INKPGPDTFP DYGDVLRAVE
Bos Taurus      CSSLTGRVIE QRIPYRGTRY PEVNRRIERL INKPGPDTFP DYGDVLRAVE
Mus musculus    CSSLTGRVIE QRIPYRGTRY PEVNRRIERL INKPGLDTFP DYGDVLRAVE
Rattus norvegicus CSSLTGKVIE QRIPYRGTRY PEVNRRIERL INKPGPDTFP DYGDVLRAVE
                         ▲
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B

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          401          ▼
Homo Sapiens    RARLQ.GTSS HSADTPEASL DSGEGPSGMA SQGCPSASRA ETDDEDDEES
Bos Taurus      RARLPQATSS HSEDTPKSSL DSGEGPSGMA SQECPTTSKA EIDDEEDEES
Mus musculus    RARLL.GTAP QPSDPPQASS ESGEGPSGMA SQECPTTSKA ETDDDDDDDD
Rattus norvegicus RARLL.ATS. QSSDLPKASS DSGEGPSGVA SQEDPTTPKA ETEDEEDDEE

          451                                              500
Homo Sapiens    DEEEEE.... .......... EEEEEEEEAT DSEEEEDLEQ MQEGQEDDEE
Bos Taurus      EEEEEEEEEE DEEEDEEEDE EDEEEEDEAT DSEEEEDLEQ MKEGQGDDEE
Mus musculus    DDDEDNEESE EE........ EEEEEEEKEA TEDEDEDLEQ LQEDQGGDEE
Rattus norvegicus SDDE...... .E........ EEEEEEEEEA TEDEDEDLEQ LQEDQDDEEE

          501                                              550
Homo Sapiens    EDEEEEAAAG KDGDKSPMSS LQISNEKNLE PGKQISRSSG EQQNKGRIVS
Bos Taurus      EE....EEAE KDGDKSPMSP LPISTEKNLE PHKGKNGSSE EQQNKRLTES
Mus musculus    EE.....GGD NEGNESPTSP SDFFHRRN.S EPAEGLRTPE GQQKRGLTET
Rattus norvegicus .E.....EGD NEDDKSPASP SPIFRRKEFS NPQKGSGPQE EQQERGLTGT
                 ▲
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Figure 5.2. Examination of evolutionary conservation of the PAH2 and D/E domains in human Daxx.
Alignment of human Daxx (gi|48146287) with corresponding Daxx sequences from *Bos Taurus* (gi|157278987), *Mus musculus* (gi|2253707), and *Rattus norvegicus* (gi|18148939) for regions around PAH2 domain (157-260) (A) and D/E region (414-505) (B). Alignment was done using using Clustal W2 (Goujon, McWilliam, et al, 2010; Larkin, Blackshields, et al, 2007). Black arrowheads indicate boundaries of regions.

In all of the *in vitro* experiments, Daxx was used without the addition of ATP. This

indicates that Daxx does not require energy to exert its refolding activity. The lack of

energy coupling seen in Daxx-mediated activity is in contrast to the energy requirement

for the traditional chaperones. While Daxx cannot suppress aggregation, and may in fact

accelerate aggregation during heat shock, it can still recover luciferase activity to a

greater proportion than when luciferase is alone or expressed with a canonical heat

shock protein (Figure 3.6). This, in combination with the observation that Daxx

accelerated denaturation of luciferase *in vitro* (Figure 3.4), suggests that Daxx can

promote a reaction that is contextually favorable, not one that is energy consuming. This

is consistent with the finding that Daxx preferentially binds to misfolded luciferase

substrates. It is important to note that while Daxx is unable to reactivate larger luciferase

aggregates *in vitro*, it is able to reactivate luciferase after heat shock *in vivo* when

luciferase should be in a mostly aggregated state (Parsell, Kowal, et al, 1994). Though

seemingly contradictory, this finding implies that either Daxx can promote the refolding of

the misfolded, not aggregated, luciferase during recovery or that it cooperates with other

chaperones to increase disaggregation.

Finally, we have shown initial evidence for a role of Daxx in the regulation of p53

distinct from what we have previously found (Tang, Qu, et al, 2006). The study by Tang,

et al also stated that Daxx does not affect the transcription of p53, thus the effect we are

seeing is most likely due to a post-transcriptional mechanism. We demonstrate that

Daxx can increase levels of p53 when Mdm2 is not present, which contradicts our

previous finding that Daxx is an indirect negative regulator of p53. We can reconcile

these two disparate ideas by the notion that Daxx is solubilizing p53 so that Mdm2 can

more easily target it for degradation or that Daxx is making the p53 ubiquitination sites

more accessible to Mdm2 via its chaperone function. The possibility cannot be excluded that since we used an Mdm2 null background, the effect we are seeing is not specific to p53 but an artifact of Daxx being able to execute its chaperone function when Mdm2 is not present, rather than participation in the Daxx-Mdm2-Hausp complex. A caveat of these experiments is that we were unable to correlate increased p53 expression with higher p53 transactivation. We attempted to perform reporter assays using the p53-responsive genes p21, Noxa, and Bax; however, we saw the repression of their transcription upon Daxx overexpression. This is expected, as Daxx is known to be a transcriptional repressor that can inhibit p53 transactivation (Gostissa, Morelli, et al, 2004), preventing us from being able to separate the effects of Daxx on transcription from those of p53 on transcription.

Future Directions

We have discovered a heretofore unknown role for Daxx in protein folding. It is clear that Daxx does not act like a traditional chaperone in that it is not able to prevent aggregation nor is it able to promote disaggregation. Rather, Daxx behaves more as an unfoldase or refoldase, favoring the thermodynamically more amenable form of a stressed substrate. We have shown both *in vitro* and *in vivo* evidence for this phenomenon but many questions remain as to how Daxx exerts its chaperone activity.

First, we have unexpectedly observed that Daxx can accelerate the denaturation of proteins during heat shock. There may be precedent for this activity as studies show that the activity of the Hsp70 chaperones can be that of an unfoldase (Sharma, De los Rios, et al, 2010; Slepenkov, and Witt, 2002). Hsp70 acts an ATP-fueled polypeptide unfoldase that acts upon misfolded monomeric substrates and traps them in an unfolded

state, after which they are released from Hsp70 and allowed to spontaneously refold without the aid of a chaperone or input of energy (Sharma, De los Rios, et al, 2010). However, this system requires the input of energy for the first unfolding step, albeit the energy cost is quite low (about 5 molecules of ATP for unfolding of one polypeptide of luciferase). Following this study, the same group discovered that certain chaperonins (GroEL-GroES, in this case) possess the same ability to unfold denatured substrates and release them into solution for spontaneous folding (Priya, Sharma, et al, 2013). When GroEL was applied to denatured rhodanese monomers, it was able to efficiently unfold rhodanese in an ATP-independent manner. Subsequent spontaneous refolding was seen as in the case of Hsp70; however, fully efficient refolding required the addition of ATP and GroES. It is tempting to postulate that Daxx may act in a similar way in that when luciferase becomes denatured it forms misfolded species that are rapidly unfolded by Daxx, as demonstrated by the acceleration of loss of activity during heat shock. After the stress is removed and/or luciferase has reached a state from which it can be refolded, it is released from Daxx and transferred to another chaperone or is able to spontaneously refold, as seen by the reactivation of luciferase during the recovery phase (Figure 3.6). To determine if Daxx is an unfoldase, we can apply similar methods used in the two studies discussed above where they use Thioflavin-T (ThT) binding to β-sheet structures as a quantifiable measure for misfolding, unfolding, and refolding. Ideally, ThT binding should be highest in the initially denatured substrate, should be lowest upon addition of the chaperone, and increase, concomitantly with an increase in activity of the substrate, during refolding.

Second, we would like to determine how Daxx recognizes misfolded substrates via the PAH2 and D/E domains. A deeper analysis of the structure of Daxx reveals many

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disordered regions (Figure 5.3) as predicted by the **P**redictor **of N**aturally **D**isordered

Regions (PONDR) VL-XT (Li, Romero, et al, 1999; Romero, Obradovic, and Dunker,

1997; Romero, Obradovic, et al, 2001). The PAH2 and D/E domains both have predicted

highly disordered regions. It is known that intrinsically disordered regions in small heat

shock proteins are involved in the binding of misfolded substrates (Haslbeck, Ignatiou, et

al, 2004; Jaya, Garcia, and Vierling, 2009; Stromer, Fischer, et al, 2004). Further, a

recent study shows that San1, a yeast nuclear ubiquitin ligase involved in protein quality

control, is able to bind to a variety of disparate substrates via its disordered N- and C-

terminal domains. These domains contain highly conserved small segments of order,

which serve as substrate-recognition sites (Rosenbaum, Fredrickson, et al, 2011). They

speculate that these disordered regions allow San1 a conformational flexibility enabling it

to bind to many different misfolded substrates. It is possible that Daxx is working through

a similar manner, which would not only explain the diverse array of Daxx binding

partners, but also its ability to bind to misfolded substrates (Figure 3.13-14). The loss of

the PAH2 and D/E domains may lead to reduced plasticity of Daxx and may explain why

neither domain alone has activity, but together they are able to renature misfolded

substrates. To test this, we could create many small deletions within Daxx and assess if

the deletion mutants have any effect on association with misfolded substrates. PONDR

predicts possible binding sites as dips in disorder; thus, it would be informative to

correlate any binding-defective mutants with regions in the PONDR plot that may be

potential binding regions.

Third, determining if the acid-rich region of Daxx defines a new function for

polyanionic entities would be enlightening as, to our knowledge, no theory has been

proposed that puts forth a biochemical function for highly acidic regions within proteins.

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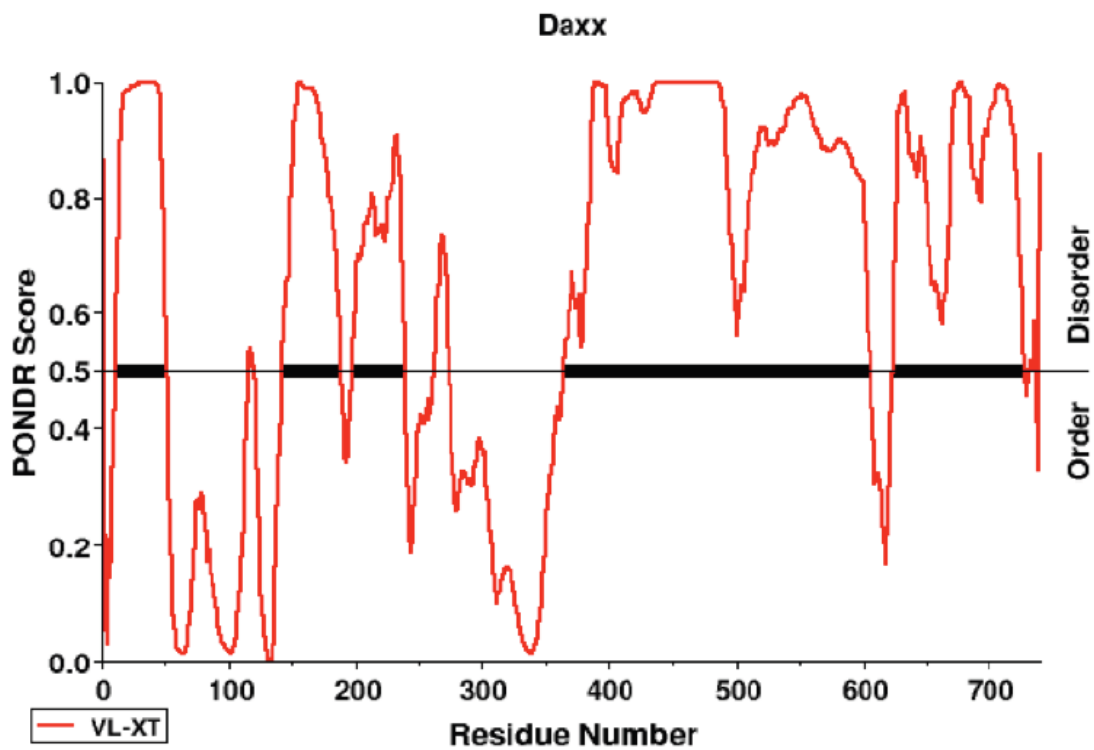
Figure 5.3. Daxx contains regions of disorder.
Disorder prediction of Daxx was run using PONDR (http://www.pondr.com). PAH2 domain lies between residues 157-260. D/E region is between residues 414-505.

Besides the ability to confer conformational flexibility as discussed above, a polyanionic stretch within a protein may have a more defined function. It has previously been shown that proteins containing polyanionic regions have chaperone activity, and that these effects are specific to anionic regions, as polycationic species do not have such activity *in vitro* (Jones, Yazzie, and Middaugh, 2004; Rentzeperis, Jonsson, and Sauer, 1999). Further, some of the smaller heat shock proteins have flexible C-terminal regions that are rich in acidic amino acids, which when mutated, lead to reduced activity (Edwards, Kueltzo, et al, 2001; Narberhaus, 2002). Members of both the large and sHsp protein families interact with polyanions such as nucleic acids and tubulin (Korber, Zander, et al, 1999; Koyasu, Nishida, et al, 1986). It is conceivable that Daxx is part of a family of acid-rich domain containing proteins that share a similar function in terms of chaperone activity. Preliminary bioinformatics analysis done by another graduate student in the lab, Aaron Stonestrom, indicated that there are about 100 proteins in the human genome that contain 40 acidic amino acids within a 100-residue window. It would be informative to test if swapping acidic regions of those proteins with that of Daxx would still allow Daxx to retain its chaperone activity, providing evidence for the generality of the polyanionic-dependence of this activity. A similar analysis was done for the PAH2 domain; however, no other proteins contained a homologous region signifying that this region is very unique and specific to Daxx.

We would also like to know if the recently determined histone chaperone function of Daxx is related to the protein folding activity we have discovered. So far, the role of Daxx as a histone chaperone has been defined as its ability to deposit the histone variant H3.3 at telomeric or pericentric chromatin in cooperation with ATRX (Drane, Ouararhni, et al, 2010; Elsaesser, and Allis, 2010; Lewis, Elsaesser, et al, 2010). A

recent study has crystallized the structure of the histone-binding domain of Daxx bound to an H3.3-H4 dimer (Elsasser, Huang, et al, 2012). They determined several single-point alanine mutations that led to disruption of the Daxx-H3.3-H4 complex: Ser220, Tyr222, Arg251, Phe317, Arg328, Asp331, and Asp349. The first three residues lie within the PAH2 domain and none of the point mutants are found in the D/E domain. It would be interesting to know how the structure of the PAH2 domain is altered in the presence of these mutations and if these mutations affect the chaperone activity of Daxx. If so, this could be a novel mode of histone chaperone activity by Daxx toward H3.3. Related to the histone chaperone activity of Daxx, mutations in Daxx are frequently found in PanNETs, often leading to an ALT phenotype. Two of these mutations, E465X and E448X are found in the D/E region and lead to nonsense mutations. Some of the other identified mutations lead to nonsense, missense, or indel mutations (Jiao, Shi, et al, 2011). It would be very intriguing to test these different mutations and their folding activity toward luciferase. If they have diminished ability to reactivate denatured substrates, this may provide a connection between histone chaperone activity, protein folding, and potentially tumorigenesis.

The molecular chaperones of the heat shock family are so named because they are responsive to heat shock and other cellular stresses (Hartl, Bracher, and Hayer-Hartl, 2011). Thus, the question arises if Daxx is also sensitive to cellular stress. Indeed, Daxx has previously been shown to be responsive to heat shock in that its SUMO-2 conjugation status is increased by 24-fold upon heat shock (Golebiowski, Matic, et al, 2009). Our results support the ability of Daxx to respond to thermal stress in that it has a preference for associating with non-native substrates formed by heat shock *in vivo* (Figure 3.13-14). Whether or not this preference is dependent upon the SUMOylation

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status of Daxx would be very interesting to determine. Currently, there is no clear

consensus as to the main function of SUMOylation in the cell, though it has been shown

to be important for transcriptional regulation, protein stability, and apoptosis

(Golebiowski, Matic, et al, 2009). The use of SUMO-modified Daxx *in vitro* may enhance

the activity of Daxx toward denatured substrates if SUMOylation is important for the

activity of Daxx. To corroborate these results *in vivo,* use of Daxx mutants that are

unable to be SUMOylated would allow us to determine the SUMO-dependence of Daxx

chaperone activity.

Finally, we have discovered that Daxx is able to solubilize misfolded p53. Our lab

has previously found that Daxx can enhance the E3 ubiquitin ligase activity of Mdm2

toward p53 both *in vitro* and *in vivo* (Tang, Qu, et al, 2006)*.* It is possible that Daxx is

promoting the oligomerization of p53, an activity important for the ability of Mdm2 to

ubiquitinate p53 (Maki, 1999). To test this, we could incubate Daxx with purified p53 and

determine the oligomerization state of p53 via gel filtration. Determining if deletion of the

PAH2 and/or D/E domain in *in vitro* ubiquitination reactions affects Mdm2 E3 activity

may suggest a link between the protein folding activity we have found with the ability of

Daxx to enhance the E3 activity of Mdm2 toward p53. Additionally, it is possible that the

phosphorylation event we have found for Daxx (see chapter two) not only leads to Mdm2

destabilization, but also allows Daxx to alter the conformation of p53 so that it can be

better targeted by Hausp or other p53 activating proteins. This could be tested via the

use of conformation-specific antibodies in the presence or absence of Daxx and with or

without DNA damage. To address more therapeutic consequences of Daxx activity, it

would be interesting to apply Daxx to conformational p53 mutants. Previous work has

indicated that some p53 mutations found in human tumors create aggregation-prone

mutant p53 that co-aggregates with wild-type p53, leading to dominant-negative effects on wild-type p53 (Xu, Reumers, et al, 2011). It is possible that Daxx is able to relieve this co-aggregation, at least insofar as wild-type p53. We have tested p53 R175H, an aggregation-prone mutant *in vitro* using mammalian-purified Daxx. We saw that Daxx was indeed able to solubilize mutant p53; however, we have not corroborated this result using Daxx purified from bacteria. To determine the *in vivo* relevance of these findings, experiments could be performed where we co-express Daxx and a p53 mutant and fractionate the cell lysate on native gels to visualize the oligomerization state of p53. It is important to note that the aggregation-prone p53 mutants generally localize in the cytoplasm, while Daxx is a predominantly nuclear protein. Thus, it would be necessary to either prevent nuclear import of Daxx or mutate putative nuclear localization signals within Daxx so that it can associate with cytoplasmic p53.

In summary, we have demonstrated that Daxx has the ability to refold misfolded proteins both *in vitro* and *in vivo*. This function may also provide a biochemical basis to unify the many functions of Daxx. Indeed, Daxx has an extensive list of binding partners, reminiscent of how Hsp70 can interact with the entire spectrum of heat-denatured proteins in *E. coli* (Mogk, Tomoyasu, et al, 1999)It is possible that Daxx acts as a general mediator of protein folding for a wide array of proteins; however, many questions remain unanswered as to the mechanism and physiological relevance of the protein folding activity of Daxx. Finding the answers to these questions would have great implications for protein quality control in the cell by determining how misfolded folded proteins are reactivated, by potentially defining a new class of chaperones containing acid-rich regions important for their activity, and by expanding histone chaperone activity to include protein folding. Additionally, determining if Daxx can solubilize pathogenic

forms of p53 would add another layer of complexity to p53 regulation by Daxx and provide a potential therapeutic target in cancer.

The Role of Daxx in Cdk6 Regulation

The cell cycle is an intricately controlled process and its regulation is necessary for the proper proliferation and growth of cells. Thus, understanding how its major effectors are controlled is important for the development of therapeutics as mutations in genes encoding the Cdks and cyclins have been observed in cancer (Vermeulen, Van Bockstaele, and Berneman, 2003). The present study showed that Daxx is able to affect the protein and mRNA levels of the cyclin D associated Cdk, Cdk6. We showed that Daxx depletion leads to lower levels of Cdk6; however, there was no robust effect on cellular proliferation. The effect of Daxx on Cdk6 was specific for Cdk6 as other cell cycle markers tested showed no difference in protein level upon Daxx knockdown. The effect on Cdk6 was also seen in a variety of cancer and primary cell types, suggesting that this was a not cell-type specific phenomenon.

A limitation of the current study was the presence of Cdk4 in all of the cell lines tested. Cdk4 and Cdk6 are generally thought to play redundant roles within the cell, though their mutual compensation has been controversial (Berthet, and Kaldis, 2006). There is only a mild phenotype associated with *Cdk6 -/-* mice but the phenotype is exacerbated in *Cdk6 -/-; Cdk4 -/-* embryos, as they die during development (Malumbres, Sotillo, et al, 2004), suggesting that Cdk4 may play a partially compensatory role when Cdk6 is not present. Thus, a more dramatic proliferative phenotype may be observed if we use cells that lack Cdk4 and then silence Daxx. If we see a strong decrease in proliferation, this would suggest that Daxx is a major regulator of Cdk6 and that this regulation has consequences for cellular proliferation.

It is also important to note that other studies have determined that loss of Daxx leads to perturbations in normal mitotic cycling (Giovinazzi, Lindsay, et al, 2012; Gongora, Stephan, et al, 2001; Kwan, Lau, et al, 2013; Pan, Yi, et al, 2013). Our results indicate that silencing of Daxx does not lead to changes in the cell cycle profile (Figure 4.11). Most of these studies observed very minute changes in cell cycle progression that were cell-type specific. It is possible that our conditions were not sensitive enough to elicit detectable changes in the cell cycle upon Daxx depletion.

Future Directions

The main challenge for us is to determine a physiologically relevant setting in which to study the regulation of Cdk6 by Daxx. We could examine the ability of Daxx to regulate Cdk6 in thymocytes as Cdk6 is thought to be the primary kinase that initiates pRb phosphorylation in thymocytes, due to its preferential expression in hematopoietic cells (Meyerson, Enders, et al, 1992; Meyerson, and Harlow, 1994). Consistent with this finding, *Cdk6 -/-* mice display defects in thymus development and reduced thymic cellularity (Malumbres, Sotillo, et al, 2004). Expanding upon these findings, another group determined that Cdk6 is required for Notch-dependent T-cell development and differentiation as cells without Cdk6 display maturation defects (Hu, Deshpande, et al, 2009). Thus, it would be interesting to determine if Daxx regulates Cdk6 in thymocytes. Furthermore, Cdk6 downregulation has been implicated in the differentiation of various cell types including osteoblasts (Ogasawara, Kawaguchi, et al, 2004) and leukemic cells (Matushansky, Radparvar, and Skoultchi, 2003). In these instances, Cdk6 expression inhibits differentiation and downregulation of Cdk6 is necessary for the cells to enter terminal differentiation steps. It would be informative to analyze Daxx levels during the differentiation of these cells; if we can observe a concomitant loss of Daxx expression,

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perhaps prior to loss of Cdk6 expression, we may be able to form a link between the importance of the regulation of Cdk6 by Daxx and cellular differentiation. Interestingly, Daxx has been shown to inhibit muscle differentiation via repression of the transcription factor, E2A (Gupta, Hou, et al, 2009); E2A inhibits Cdk6 gene expression (Schwartz, Engel, et al, 2006). This suggests a model whereby inhibition of E2A by Daxx allows for Cdk6 activation and inhibition of cell differentiation. We should determine the E2A-dependence of the Daxx effect on Cdk6 via silencing of E2A in cells.

We were unable to go far beyond mRNA analysis (Figure 4.8) and promoter binding (Figure 4.9) to determine the role of Daxx in *Cdk6* transcription. Since Daxx is not known to contain a DNA binding domain, it is likely that Daxx is indirectly binding to the *Cdk6* promoter. There are binding sites for NF-κB, Ets, Sp1, and p300 within the *Cdk6* promoter (Cram, Liu, et al, 2001). Daxx is known to modulate the transcriptional activity of NF-κB and Ets; thus, it would be informative to test if experimental reduction of either transcription factor would abrogate the transcriptional regulation of Daxx toward Cdk6.

Another interesting question is if Daxx works in cooperation with ATRX to control Cdk6 transcription. Importantly, U2OS cells do not contain endogenous ATRX (Niforou, Anagnostopoulos, et al, 2008), yet we were able to observe a decrease in Cdk6 levels upon Daxx knockdown (Figure 4.3A), suggesting that Daxx may not be completely dependent upon ATRX for its activity. It is still possible that in other cell lines, Daxx and ATRX work together to repress a subset of genes, one of which may be Cdk6. Daxx and ATRX are known to form a heterochromatin-remodeling complex, which is able to regulate transcription often via recruitment to sequence-specific transcription factors that bind to these promoters (Tang, Wu, et al, 2004; Xue, Gibbons, et al, 2003). When ATRX

is tethered to DNA, it is able to repress transcription. However, ectopic expression of Daxx alleviates ATRX-mediated transcriptional repression via sequestration of ATRX to PML-NBs by Daxx (Tang, Wu, et al, 2004). It is possible that the effect we are seeing is partially ATRX-dependent and that ATRX can also inhibit Cdk6 transcription. Theoretically, when Daxx is not present, ATRX is able to repress transcription of Cdk6; however, when Daxx is present, ATRX is sequestered to PML-NBs, thereby alleviating the transcriptional repressive effects of ATRX toward Cdk6.

Concluding Remarks

This dissertation has examined several biological functions of Daxx. We have shown that Daxx is a novel substrate of ATM and that phosphorylation of Daxx by ATM impairs the association of Daxx and Mdm2, allowing for p53 activation. Second, we have shown that, depending upon the cellular context, Daxx has the ability to promote the denaturation or renaturation of a protein. Finally, we have identified a new transcriptional target of Daxx: Cdk6, a cyclin D associated kinase involved in both cellular proliferation and differentiation. While these findings further our knowledge regarding the intracellular activities of Daxx, they also raise many questions as to how and when Daxx exerts these activities. Creation of a conditional *Daxx -/-* mouse may help answer some of these questions if defects in cellular functions are found and/or if these defects occur in a tissue-specific manner. Additionally, elucidation of the crystal structure of Daxx would allow us to determine how Daxx preferentially binds to misfolded species and where exactly this interaction occurs.

Its demonstrated flexibility in response to different cellular conditions forces us to question why the role of Daxx would be so multifunctional. It is possible that Daxx can mediate a set of diverse responses depending upon the needs of the cell, providing a

potential explanation for its extensive list of interacting partners. The novel role of Daxx in protein folding may also provide a biochemical mechanism to unify all these functions of Daxx in that Daxx can assist a wide range of proteins to reach their native conformation; this may be why Daxx is involved in such disparate activities. Further, Daxx has been implicated in a few instances of human disease; however, the role of Daxx in the pathogenesis of disease remains unclear. Understanding the dynamic nature of Daxx will help us not only to understand the basic regulation of many biological processes, but may also provide a basis for establishing Daxx as a valid therapeutic target.

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