GATEKEEPERS OF THE GUARDIAN: P53 REGULATION BY POST-TRANSLATIONAL MODIFICATION, MDM2 AND MDMX

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INTRODUCTION

Happy 25th Anniversary p53! Since this is such a special occasion, I (GW) thought of explaining how fate brought p53 and me together.

It was a snowy day in Utah when Arnie Levine came to the University of Utah in 1976 to present a lecture on genetic approaches to differentiation of teratocarcinoma cells. My graduate work with Mario Capecchi (starting at Harvard and continuing at the University of Utah) led me to appreciate the potential power of genetics in cancer research. I therefore arranged to visit Arnie's lab to learn more about his research program. We discussed many topics, but not about how the large transforming protein (T antigen) of SV40 (SV40TAg) interacted with a putative ~54kDa cellular protein (Linzer and Levine, 1979).

I next went to Stanford to visit George Stark, whose lab had isolated mutant cancer cell lines resistant to PALA, an inhibitor of *de novo* uridine synthesis. Unlike most mutants described to that time, PALA resistance developed incrementally, and was associated with progressive increases in the levels of CAD, the enzyme targeted by the drug. Molecular cloning was just starting at Stanford, and I could see a clear route to solving the genetic mechanism(s) of this unusual form of drug resistance in cancer cells. I joined George's group in January 1977, and soon met a sabbatical visitor named Lionel Crawford. Lionel told me about work his post-doc, David Lane, was doing with SV40, and how SV40TAg associated with an ~54kDa protein, presumably of cellular origin (Lane and Crawford, 1979). I thought this was an interesting curiosity, but I didn't see the links to cellular transformation at that time. I certainly couldn't imagine at that early date how SV40TAg interactions with cellular proteins might relate to understanding the mechanisms of PALA resistance.

My studies in George's lab with a graduate student, Richard Padgett, showed that gene amplification was the sole mechanism accounting for PALA resistance in the cell lines we investigated (Wahl et al., 1979). When I left Stanford, I recall having a discussion with George in which I asked him whether normal cells treated with the same drug also acquired PALA resistance. He answered that they had just done one experiment to address this question, and he told me something that would affect my research program for the next decade: he said that normal cells appeared to stop dividing, while cancer cells died when treated with equivalent PALA concentrations. My interpretation of his comment was that normal cells have controls that prevent them from cycling in response to this drug, while cancer cells may have lost such controls. Thus, my objective for the future was to explore the validity of this hypothesis, and to try to elucidate genes involved in the control circuitry.

Fast-forward a decade. Work from my lab and others showed that DNA breakage initiates gene amplification, and that breakage is induced when cells enter and proceed through S-phase under nucleotide-limiting conditions (Morgan et al., 1986; Windle et al., 1991). I therefore started to look for genes that prevent cells from entering S-phase under conditions that induce chromosome breakage. An exciting candidate emerged after I read some papers from Mike Tainsky's group in which *in vitro* passage of cells derived from Li-Fraumeni patients led to chromosome abnormalities similar to those in cells undergoing gene amplification (compare Bischoff et al., 1990; Morgan et al., 1986; Windle et al., 1991). As Li-Fraumeni patients have germ line p53 mutations, we began to investigate whether there was a link between p53 loss and gene amplification. More specifically, we predicted that normal cells treated with PALA would arrest prior to S-phase, while p53-deficient cells would enter S-phase, undergo chromosome breakage, and generate rare survivors with amplification of the CAD gene (which gives rise to PALA-resistance by enabling over-expression of the CAD protein (Yin et al., 1992). In retrospect, the seminal work of Michael Kastan and colleagues linking p53 to a G1 damage checkpoint (Kastan et al., 1991) makes this a logical expectation.

It was a memorable day when a post-doc in my lab, Yuxin Yin, excitedly showed me the results that corroborated our hypothesis linking loss of p53 to failed cell cycle control and gain of amplification competence (Yin et al., 1992). Interestingly, in contrast to the extensive death PALA induced in cancer cell lines, we noticed that a significant fraction of a normal cell culture treated with PALA re-entered the cell cycle when the drug was removed. We later showed this was due to the ability of ribonucleoside depletion to cause cells in G1 to activate p53, which prevents them from entering S-phase and undergoing breakage (Linke et al., 1996). These studies demonstrated p53

could serve as a "Guardian of the Genome" (term attributed to David Lane; Lane, 1992) by its ability to halt cell cycle progression in response to conditions that could induce genetic instability, such as DNA damage or ribonucleoside depletion; conversely, our work also demonstrated that loss of p53 enabled tumor cells to proliferate under DNA damaging conditions to generate genetically unstable variants (Yin et al., 1992). Similar conclusions were reached by work performed independently in the labs of Thea Tlsty and George Stark (Livingstone et al., 1992; Perry et al., 1992).

Twenty-five years later, more than 30,000 articles have been published on the small cellular ~54kDa protein bound by SV40T-antigen and the increasing number of proteins that regulate it. It is clear that this protein, now referred to as p53, is a tumor suppressor gene (Malkin et al., 1990; Srivastava et al., 1990) that is inactivated by mutation in about half of all human cancers (Hollstein et al., 1991). A substantial fraction of the remaining cancers have functionally compromised p53 due to alterations in its regulators such as MDM2 and the related protein MDMX (e.g., Momand et al., 1998; Riemenschneider et al., 1999). Below, we will refer to the mouse and human homologs (Migliorini et al., 2002a) as MDM2 and MDMX for simplicity.

MECHANISMS OF P53 MEDIATED TUMOR SUPPRESSION

Why is p53 so frequently inactivated in cancer? The answer likely relates to the ability of p53 to eliminate cells that encounter conditions that could induce genetic instability or promote unscheduled cell division. We now know that p53 is activated by small amounts of various types of DNA damage (e.g., see Huang et al., 1996; Kastan et al., 1991; Wahl and Carr, 2001), short or abnormally structured telomeres (Chin et al., 1999; Karlseder et al., 1999), metabolic and other consequences of high level oncogene signaling (Denko et al., 1994; Felsher and Bishop, 1999; Mai et al., 1996; Sherr, 2001; Vafa et al., 2002), microtubule dysfunction (Di Leonardo et al., 1997; Khan and Wahl, 1998; Lanni and Jacks, 1998; Minn et al., 1996), loss of nucleolar integrity (Rubbi and Milner, 2003), hypoxia (Alarcon et al., 1999) and perturbation of the endoplasmic reticulum (ER) (Qu et al., 2004). The list has grown continuously over the years, so it wouldn't be surprising if more p53 activating conditions were identified in the future.

The mammalian p53 pathway generates responses as varied as reversible cell cycle arrest and apoptosis based on the nature of the activating signal and cell type. Since p53 output can kill cells, stringent regulatory mechanisms must have evolved to prevent its errant activation, as well as to allow it to rapidly initiate a response when appropriate. We will review studies that are starting to provide insight into how the p53 regulatory circuit evolved to control genetic stability, the cell cycle, and apoptosis to limit tumor formation. We

will analyze *in vitro* and *in vivo* data that raise questions about the contributions of highly conserved phosphorylation sites to p53 control, and how mouse models are indicating that these sites may only be important in specific tissues. Finally, we will discuss recent studies describing an important new contribution to p53 control: the requirement for DNA damage to induce the degradation of MDM2 to activate p53.

p53 suppresses tumor formation largely by transcriptional regulation of a diverse set of target genes, but the importance of transcription-independent mechanisms is still being investigated (see below). p53 binds degenerate consensus sequences consisting of two inverted repeats in each half-site (el-Deiry et al., 1992; Funk et al., 1992). It binds most efficiently to its response elements as a tetramer, but the binding efficiencies and kinetics are likely affected by factors such as the precise sequence of the response elements, the type of other regulatory elements in the control region of the target gene, and chromatin context (Espinosa et al., 2003; Friedman et al., 1993; Inga et al., 2002; McLure and Lee, 1998; Szak et al., 2001). Most p53 mutations in human cancers affect the structure of its large DNA binding domain, or the residues used to contact the DNA backbone (see Cho et al., 1994; Gorina and Pavletich, 1996). These data imply that effective tumor suppression requires that p53 contact its response elements in chromatin.

Control of cell cycle arrest

Many mechanisms have been suggested to account for p53-mediated tumor suppression, but its abilities to induce cell cycle arrest or apoptosis and as a consequence to prevent unscheduled proliferation and to limit genetic instability appear paramount. Each of these functions can be largely accounted for by transcriptional activation of appropriate target genes. For example, p53's ability to induce a G1 arrest in response to DNA damage mainly depends on induction of the cyclin-cdk inhibitor p21/waf1/cip1/sdi1 (el-Deiry et al., 1993; Noda et al., 1994). One piece of evidence supporting this conclusion is that p21 deletion in mice and in human cell lines almost entirely prevents DNA damage from inducing a G1 arrest (Deng et al., 1995; Waldman et al., 1995). Consistent with the data in mammals, the p53 ortholog in Drosophila is activated by DNA damage, but it does not induce the p21-like gene Dacapo, and consequently does not induce a cell cycle arrest (Ollmann et al., 2000). p53 also participates in G2 arrest through induction of 14-3-3 sigma and GADD 45 (for examples, see (Hermeking et al., 1997; Jin et al., 2000; Yang et al., 2000). GADD45 function is required for efficient G2 arrest induced by base-alteration mutagens but not ionizing radiation (Hollander et al., 1999), while p21 helps to sustain G2 arrest triggered by DNA damage (Bunz et al., 1998).

Control of apoptosis

The mechanisms by which p53 regulates apoptosis continue to be debated. A strong case can be made for transcription-dependent mechanisms as p53 regulates many pro-apoptotic genes including BAX, PUMA, PERP, NOXA, AIP1, FAS1/APO1, and IGF-BP3 in mammals and, hid, sickle, EIGER and reaper in Drosophila (see Wahl and Carr for mammalian references; representative fly references are Brodsky et al., 2000; Lee et al., 2003; Peters et al., 2002). Target gene activation by fly p53 is required for DNA damage-induced apoptosis since combined deletion of hid, sickle and reaper abrogates the apoptotic response in flies with wild type p53 (Brodsky et al., 2004). It is likely that apoptosis regulation in mammals is more complex, with different genes or gene sets being determined by the cell type and activating stimulus. For example, BAX appears to be a key gene for inducing apoptosis by p53 in an oncogene (Eμ-Myc) model of lymphomagenesis (Eischen et al., 2001), whereas BAX loss only partially reduces DNA damage-induced apoptosis in E1A expressing MEFs (McCurrach et al., 1997). PUMA has recently emerged as a critical p53 pro-apoptotic BH3-only target gene in several tissues since PUMA knockout mice are completely deficient in damage-induced apoptosis in the CNS and thymus (Jeffers et al., 2003; Villunger et al., 2003). The tissue and gene-specific requirements complicates the problem of defining the transcriptional targets and transcriptional-dependence of p53-activated apoptotic programs.

On the other hand, it has long been debated whether the sole mechanism by which p53 induces apoptosis involves transcriptional regulation (Caelles et al., 1994; Chipuk et al., 2004; Mihara et al., 2003; Moll and Zaika, 2001). Recent papers suggest that p53 can interact with apoptotic regulators in the cytoplasm to induce an apoptotic program without gene activation, but the specific interactions do not seem consistent in different studies (Chipuk et al., 2004; Mihara et al., 2003). For example, one study showed that p53 may interact anti-apoptotic proteins such as BCL2 to liberate BAX and BAK (Mihara et al., 2003), while another reported that direct interaction between p53 and BAX enabled BAX to associate with mitochondria to induce cytochrome C-release (Chipuk et al., 2004). The latter study also showed that mouse embryo fibroblasts encoding a transcriptionally inactive and nuclear restricted endogenous $p53^{QS}$ allele (Jimenez et al., 2000) could be made to undergo apoptosis by using wheat germ agglutinin to accumulate $p53^{QS}$ in the cytoplasm (Chipuk et al., 2004). However, the use of wheat germ agglutinin to force cytoplasmic accumulation could sensitize the cells to apoptotic signals since it blocks nuclear import of proteins, and nuclear export of proteins and RNA (Middeler et al., 1997; Watanabe et al., 1999; Yoneda et al., 1987). The resulting macromolecular mislocalization might enable

cytoplasmic p53 to tip the balance towards apoptosis. For example, treatment of cells with the nuclear export inhibitor leptomycin B (LMB) creates a stress that activates p53 and can induce apoptosis (Smart et al., 1999). The biological significance of a cytoplasmic component for p53- induced apoptosis is also uncertain as the cytoplasmic abundance of p53 is highest in unstressed, exponential cells and p53 is almost exclusively nuclear when cells are exposed to apoptotic stresses (see Shirangi et al., 2002; Stommel and Wahl, 2004 for recent analyses). Quantifying the contributions of transcriptional and non-transcriptional mechanisms for p53-induced apoptosis will require analysis of p53 mutants that are transcriptionally inactive and cytoplasmically sequestered that are expressed at normal levels.

Control of genetic stability

p53 has been reported to limit genetic instability in two broad ways. First, p53 can prevent cells with irreparable lesions from proliferating by inducing a permanent arrest resembling senescence or apoptosis (see Wahl et al., 1997) for a review). Second, depending on the type of damage induced, the cell cycle phase in which p53 is activated, and the cell type, p53 increases repair efficiency by inducing cell cycle delays, activating repair genes, or participating directly in some forms of repair. For example, p53-induced expression of the cyclin-dependent kinase inhibitor p21 can modulate the G2/M interval in response to ionizing radiation (Bunz et al., 1998). This may limit instability by allowing additional time for the cell to repair double strand breaks. Conversely, p21 deficiency increases the chance that cells with unrepaired chromosomes will enter G1 to generate descendants with chromosome anomalies (Bunz et al., 1998; Wouters et al., 1997). Consistent with this, irradiated cells deficient in p53 or p21 progressed more rapidly through G2/M-phase. This resulted in increased chromosome anomalies, cell death, and sensitization to radiation (Bunz et al., 1998; Wouters et al., 1997). The G2 delay may increase double strand break repair due to the availability of the sister chromatid as a template for homologous recombination and error-free repair.

Repair of double strand breaks by homologous recombination is not likely in G1 as the sister chromatid is not present (see Wahl and Carr, 2001) for review). Therefore, double strand breaks are repaired in G1 by an error-prone process such as non-homologous end joining (Lees-Miller and Meek, 2003). p53 limits the probability of cells with unrepaired DNA in G1 from generating mutant offspring by inducing a permanent arrest or apoptosis to remove such cells from the proliferating pool (Wahl and Carr, 2001). The importance of p53 for policing the repair process is vividly illustrated in analyses of cells deficient in enzymes that participate in non-homologous end

joining or the histone γH2AX that either protects broken ends or organizes the chromatin to optimize repair efficiency. Cells deficient in these proteins are hyper-sensitive to breakage, which can lead to lethality in mice with wild type p53 (Bassing and Alt, 2004; Bassing et al., 2003; Gao et al., 2000). Loss of p53 rescues the lethality, but makes the animals tumor prone due to an increased rate of accumulation of chromosome abnormalities such as oncogene amplification that can drive tumor progression (Bassing et al., 2003; Gao et al., 2000).

p53 has also been reported to modulate the DNA repair process by transcription-dependent and transcription-independent mechanisms. For example, p53 modulates the repair efficiency of base DNA damage induced by UV and ionizing radiation through induction of genes such as DDB2 (p48) (Fitch et al., 2003; Hwang et al., 1999), GADD45a (Smith et al., 2000) and XPC (Amundson et al., 2002). The proteins encoded by these genes participate in the global genomic repair subpathway of nucleotide excision repair. p53 may also participate directly in base excision repair to correct damage induced by alkylating agents such as MMS (Offer et al., 1999; Seo et al., 2002; Zhou et al., 2001b). In this case, interactions between p53, apurinic endonuclease, and DNA polymerase beta appear to be important (Zhou et al., 2001b). p53 has been proposed to be able to facilitate repair through a putative strand annealing function and an intrinsic 3'-5' exonucleolytic activity in the DNA binding domain (Janus et al., 1999). The relative importance of p53-mediated transcriptional and non-transcriptional mechanisms to DNA repair and the control of genomic stability remain to be determined.

REGULATING p53

p53 must be tightly regulated as it has the potential to either kill a cell or to prevent it from dividing again. It is likely that most of this control is through post-translational regulation that activates or suppresses p53 as a transcription factor. Strong evidence that p53 mediated tumor suppression requires a functional transactivation domain was obtained using homologous recombination to generate cell lines or mice encoding a transcriptionally inert p53 protein (p5325Q26S=p53QS) (Chao et al., 2000b; Jimenez et al., 2000). This p53 mutant binds to its consensus sequences in EMSA (*ibid*.) and ChIP assays (M. Tang and G. Wahl, unpublished), but fails to induce or repress known target genes as the two amino acid changes it contains prevents interaction with the basal (Lu and Levine, 1995) transcription machinery (Thut et al., 1995; Xiao et al., 1994) . It does not elicit apoptosis or cell cycle arrest *in vitro* or *in vivo* (Chao et al., 2000b; Jimenez et al., 2000; M. Nister, M. Tang, M. Beeche, T. van Dyke, G..M. Wahl, manuscript in preparation).

Importantly, mice with this mutation exhibit the same tumor spectrum and latency as animals completely lacking p53 protein (M. Nister, M. Tang, M. Beeche, T. van Dyke, G..M. Wahl, manuscript in preparation). These data, along with others summarized above, indicate that nuclear, presumably transcription-dependent, functions of p53 are critical for it to suppress tumor formation. Therefore, the remainder of this chapter will focus on the factors and mechanisms that regulate the nuclear functions of p53.

A small digression concerning MDM2 is necessary to enable a discussion of p53 control mechanisms (see below for more thorough discussion of MDM2). p53 levels are kept low mainly through the combined actions of two related RING finger proteins, MDM2 and MDMX (MDM4), that can associate as homo- or heterodimers through their RING domains (Ashcroft and Vousden, 1999; Gu et al., 2002; Haupt et al., 1997; Kubbutat et al., 1997; Michael and Oren, 2003; Migliorini et al., 2002a; Sharp et al., 1999; Tanimura et al., 1999). MDM2 was first identified as the 90kDa protein encoded by a gene amplified on mouse "double minute (DM) chromosomes" and has since been observed to be amplified in a subset of human tumors expressing wild type p53 (Oliner et al., 1992). Overexpression of MDM2 can prevent p53 induced cell cycle arrest and apoptosis (Chen et al., 1994; Oliner et al., 1993). Similarly, MDMX is the likely target gene in the 1q32 amplicon detected in a subset of gliomas with wild type p53 (Riemenschneider et al., 1999). Thus, MDM2 and MDMX appear to be oncogenes in human cancers. Both genes are also essential since deletion of either leads to early embryonic lethality in mice (Jones et al., 1995; Migliorini et al., 2002c; Montes de Oca Luna et al., 1995; Parant et al., 2001). Importantly, deleting p53 eliminates the lethality of MDM2 or MDMX deficiency (*ibid.*). These data establish p53 as the key downstream target of MDM2 and MDMX, and MDM2 and MDMX as essential negative regulators of p53.

MDM2 is a ring finger E3 ubiquitin ligase that mediates the ubiquitination and degradation of p53 (Fang et al., 2000; Fuchs et al., 1998; Haupt et al., 1997; Honda et al., 1997; Honda and Yasuda, 2000; Kubbutat et al., 1997; Lai et al., 2001). MDM2 mediated ubiquitination mainly occurs on C-terminal lysines, and transfection analyses show that p53 mutants in which all these lysines were changed to arginine are stable, active and nuclear (Nakamura et al., 2000; Rodriguez et al., 2000). Other studies show that p53 mutations that prevent MDM2 association also generate stable, nuclear p53 (Jimenez et al., 2000; Lin et al., 1994). These studies establish links between MDM2 interactions with p53, p53 protein abundance, and p53 subcellular localization.

Subcellular localization

p53 is a very unstable protein that is typically nuclear and present at very low levels. p53 appears to shuttle between nucleus and cytoplasm during the cell cycle (David-Pfeuty et al., 1996; Moll et al., 1996; Ostermeyer et al., 1996; Shaulsky et al., 1991); its nuclear entry and exit are mediated by specific import and export machinery as it exceeds the 40-50kDa limit for passive nuclear shuttling (Gorlich and Kutay, 1999). p53 contains nuclear localization and nuclear export signals, and its subcellular localization reflects a balance between the rates of import and export (Henderson and Eleftheriou, 2000; Shaulsky et al., 1990; Stommel et al., 1999; Zhang and Xiong, 2001). Given the importance of nuclear functions of p53 in tumor suppression, it is not surprising that some tumors have evolved mechanisms to accumulate p53 in the cytoplasm to inactivate it (e.g., see Moll et al., 1995; Moll et al., 1992; Sun et al., 1992).

p53 has two reported nuclear export signals (NES), a C-terminal one within the tetramerization domain (Stommel et al., 1999), and a second that overlaps the N-terminal transactivation domain (Zhang and Xiong, 2001). Because treatment of cells with leptomycin B (LMB), which inhibits the nuclear export receptor CRM1 (Kudo et al., 1998; Wolff et al., 1997), results in p53 nuclear localization, either or both are potential CRM1 targets (Stommel et al., 1999).

The C-terminal NES has the potential to link p53 structure with subcellular localization and nuclear functions. The crystal structure of the tetramerization domain indicates that the NES it contains should be concealed in the tetramer, but exposed in monomers or dimers. The importance of the C-terminal NES for controlling p53 subcellular localization is indicated by the p53 nuclear restriction caused by C-terminal NES mutations (Stommel et al., 1999). Thus, the positioning of an NES in the tetramerization domain allows for factors that affect p53 tetramerization and dissociation to be linked to subcellular localization and binding of p53 to its response elements. As an example, some studies indicate that phosphorylation of serine 392 (human p53) in the C-terminus might stabilize p53 tetramers, while phosphorylation of serines 315 and 392 might destabilize tetramers (Sakaguchi et al., 1997; see Jimenez et al., 1999; Liang and Clarke, 2001 for reviews). Other studies have been interpreted to indicate that MDM2-mediated ubiquitination of p53 may expose the p53 C-terminal NES to enable p53 export to the cytoplasm (Boyd et al., 2000; Geyer et al., 2000; Lohrum et al., 2001).

The N-terminal NES has also been proposed to induce p53 nuclear export. This NES is proposed to be active in unstressed cells, but is inactivated by DNA damage to allow for rapid nuclear accumulation (Zhang and Xiong, 2001). However, this putative NES lies within the transactivation domain, and overlaps the sequences known to bind MDM2 (see Michael and Oren, 2003)

for a recent MDM2 review and references). While DNA damage induced N-terminal phosphorylations were proposed to inactivate the N-terminal NES (Zhang and Xiong, 2001), these modifications occur in regions that could affect MDM2-p53 association (e.g., see Dumaz et al., 2001; Shieh et al., 1999; Siliciano et al., 1997; Unger et al., 1999a). Also, DNA damage also induces modifications on MDM2 (Maya et al., 2001) that can reduce MDM2 stability (Stommel and Wahl, 2004), which also impedes MDM2-p53 interaction. Furthermore, mutations in the proposed N-terminal NES designed to limit interaction with the export receptor were made in residues that prevent association with MDM2 (Kussie et al., 1996; Lin et al., 1994). Consequently, these mutations stabilize p53, leading to its tetramerization, and constitutive nuclear localization (Jimenez et al., 2000; Stommel et al., 1999). Thus, the data used to support the existence of an N-terminal NES can also be explained by the fact that each treatment or condition antagonizes MDM2 binding, leading to increased p53 abundance, tetramerization and masking of the C-terminal NES. Alternatively, conditions that reduce MDM2-p53 association should also reduce p53 C-terminal ubiquitination, which could also reduce p53 nuclear export by preventing unmasking of the C-terminal NES (see above).

As mentioned above, a number of cancer cell lines have cytoplasmic p53. Several mechanisms could account for this. First, these cells could have mutations that lead to an excess of p53 nuclear export over import. In support of this idea, treatment of neuroblastoma cells exhibiting cytoplasmic p53 with either p53 C-terminal peptides that bind the C-terminal NES or with the export inhibitor LMB (Ostermeyer et al., 1996; Smart et al., 1999; Stommel et al., 1999) leads to nuclear accumulation of p53. Another mechanism for cytoplasmic accumulation of p53 involves association with a cytoplasmic anchor protein. One candidate for such a molecule is Parc (p53-associated parkin-like cytoplasmic protein; Nikolaev et al., 2003). This large protein is overproduced in neuroblastomas with cytoplasmic p53, and reducing Parc in these cells by siRNA induced p53 nuclear localization, apoptosis and sensitization to chemotherapy (Nikolaev et al., 2003). Interestingly, the same neuroblastoma cells in which elevated Parc was proposed to bind to p53 and sequester it in the cytoplasm were shown previously to exhibit hyperactive p53 export (Ostermeyer et al., 1996; Smart et al., 1999; Stommel et al., 1999). The basis for these different results remains to be determined, However, it is uncertain how much of a role Parc plays in controlling subcellular localization of p53 in normal, unstressed cells as they contain very low levels of mainly nuclear p53.

MDM2 and MDMX as inhibitors of p53 transactivation

MDM2 inhibits p53 function in at least two ways, though it apparently needs MDMX to do so with optimal efficiency (Gu et al., 2002; Migliorini et al., 2002a). First, similar N-terminal regions of MDM2 and MDMX interact with hydrophobic side chains of an amphipathic alpha-helix in the p53 N-terminal transactivation domain (Bottger et al., 1999; Chen et al., 1993; Kussie et al., 1996). Consequently, MDM2 and MDMX, by binding to the transactivation domain, could inhibit transactivation by competing with the basal transcription machinery for binding and/or by preventing p53 acetylation by histone acetyl transferases such as p300 and CBP (Gu et al., 1997; Lu and Levine, 1995; Momand et al., 1992; Oliner et al., 1993; Shvarts et al., 1996; Thut et al., 1995; Xiao et al., 1994). MDM2 may also inhibit p53 transactivation by recruiting co-repressors such as CtBP2 or titrating basic transcription factors (Mirnezami et al., 2003; Thut et al., 1997).

Regulating p53 stability by MDM2 and MDMX

The second mechanism by which MDM2 inhibits p53 is by acting as a co-factor for p53 degradation. p53 was initially shown to be targeted for degradation by the oncogenic papilloma virus E6 protein, which binds to p53 and recruits a cellular ubiquitin ligase (E6-AP) to mediate p53 ubiquitination (Scheffner et al., 1993). Support for a completely host-encoded mechanism for p53 proteasomal turnover was indicated by the substantial increase in ubiquitinated p53 caused by the proteasome inhibitor MG132 in cells that were not virally infected (Maki et al., 1996).

The MDM2 ubiquitin ligase that mediates p53 degradation (see above) also mediates its own poly-ubiquitination (Lai et al., 2001). However, there is debate about whether MDM2 mediates the mono- (Lai et al., 2001), or poly-ubiquitination of p53 (Li et al., 2003). This debate was sparked by a recent report showing that low levels of MDM2 mediate p53 mono-ubiquitination, while higher levels induce poly-ubiquitination (Li et al., 2003). This is an important distinction as mono-ubiquitination can mediate changes in subcellular distribution but not degradation, while chains containing at least four ubiquitins are required for proteasomal degradation (Thrower et al., 2000; see Hicke, 2001 for a review). Indeed, p53 mono-ubiquitination was reported to lead to nuclear export of p53, while poly-ubiquitination led to p53 degradation (Li et al., 2003). Either consequence of MDM2-mediated ubiquitination would diminish p53's capacity to regulate gene expression.

The notion that MDM2 could induce mono-ubiquitination for p53 export or polyubiquitination for degradation raises the question of whether p53 must be exported to be degraded in the cytoplasm (Boyd et al., 2000; Freedman and Levine, 1998; Geyer et al., 2000; Inoue et al., 2001; O'Keefe et al., 2003; Roth et al., 1998; Tao and Levine, 1999). The model summarized above

predicts that in unstressed cells, MDM2 should be at low levels, leading to p53 mono-ubiquitination, nuclear export, and cytoplasmic accumulation. However, in unstressed cells, p53 is present at low abundance but is predominantly nuclear (e.g., see Stommel and Wahl, 2004). It is also important to consider that p53 half-life is about 30 minutes in unstressed cells (e.g., see Oren et al., 1981; Stommel and Wahl, 2004), while its export to the cytoplasm takes hours as the C-terminal p53 NES is very weak (Henderson and Eleftheriou, 2000; Stommel et al., 1999). The slow export rate and short half-life are incompatible with models requiring that p53 export is required for its degradation in the cytoplasm. As proteasome inhibitors lead to p53 accumulation in the nucleus and the cytoplasm, it appears to be unstable in both locations (see Stommel and Wahl, 2004 for a recent example). Consistent with this interpretation, nuclear and cytoplasmic p53 can be ubiquitinated and degraded, implying that proteasomes in both compartments accept it as a substrate (Geyer et al., 2000; Joseph et al., 2003; Lohrum et al., 2001; Shirangi et al., 2002; Stommel and Wahl, 2004; Xirodimas et al., 2001; Yu et al., 2000). However, the slow export kinetics of p53, and its co-localization with MDM2 in the nucleus, suggest the nucleus as a preferred site for p53 turnover.

The precise mechanisms by which MDM2 leads to p53 degradation remain to be defined. p53 polyubiquitination might be achieved by MDM2 alone in cells expressing high levels of MDM2, such as in cancers with MDM2 amplification or overexpression. This begs the question of how poly-ubiquitination is achieved in normal cells with unstable p53 and low levels of MDM2, since MDM2 may only induce mono-ubiquitination under such conditions (Fang et al., 2000; Li et al., 2003). One solution is that MDM2 could associate with another ubiquitin ligase, an "E4", that adds poly-ubiquitin chains to the lysines previously mono-ubiquitinated by MDM2. This interaction is likely to involve the MDM2 RING domain, since replacing it with the RING domain of another protein, Praja1, enables MDM2 to poly-ubiquitinate itself but prevents p53 ubiquitination (Fang et al., 2000). However, this situation may be more complex as the MDM2 RING domain has also been implicated in other processes such as ATP binding and acetylation that may also affect MDM2 function (Poyurovsky et al., 2003; Wang et al., 2004). The central region of MDM2 containing an acidic domain is also needed for p53 degradation (Argentini et al., 2001; Kawai et al., 2003b; Meulmeester et al., 2003). Interestingly, hHR23A a human homologue of a yeast DNA repair protein, binds to the 26S proteasome and to the acidic domain of MDM2 (Hiyama et al., 1999; Zhu et al., 2001a; Brignone et al., 2004).

One potential candidate for a p53 E4 polyubiquitin ligase is the histone acetyl transferase p300 (Grossman et al., 2003). At first glance, this seems to be a surprising finding, since p300 binds to the same N-terminal region of p53 as MDM2, it acetylates the same C-terminal lysines that MDM2 mono-ubiquitinates, and it has been reported to be a p53 co-activator (Barlev et al., 2001; Gu and Roeder, 1997). However, consistent with a role for p300 in p53 degradation, MDM2, p300 and p53 form ternary complexes, and MDM2 mutants that cannot bind p300 can mediate p53 ubiquitination but not degradation (Grossman et al., 1998; Kobet et al., 2000; Zhu et al., 2001b). It is also possible that p300 serves as a bridge to either the proteasome, to other ligases that mediate polyubiquitination, or to proteins that associate with the proteasome, such as hHR23A binds to p300 (Zhu et al., 2001a).

The precise mechanisms by which MDM2 and MDMX collaborate to regulate p53 are important to define, as both proteins are clearly required for optimal inactivation of p53. MDM2 mediates the ubiquitination and degradation of MDMX (Kawai et al., 2003a; Pan and Chen, 2003; Tanimura et al., 1999). On the other hand, MDMX cannot induce MDM2 degradation as it is not an E3 ubiquitin ligase (Stad et al., 2001). Several studies employing transfection and overexpression indicated that MDMX can inhibit p53 degradation (Jackson and Berberich, 2000; Sharp et al., 1999; Stad et al., 2001). This is contrary to genetic analyses in mice and more recent siRNA studies showing that MDMX depletion activates and stabilizes p53 (Kawai et al., 2003a; Migliorini et al., 2002b; Parant et al., 2001). These disparate observations now seem to be resolved by a study that changed the ratio of MDMX relative to MDM2 and then determined the effects on p53 degradation. These studies showed that MDMX can stabilize MDM2, and when present at an appropriate ratio, MDMX increases significantly the ability of MDM2 to degrade p53 (Gu et al., 2002). These data explain how both MDMX and MDM2 assist each other to maximize p53 inhibition, and how deletion of either elicits embryonic lethality. If MDM2 is deleted, MDMX may bind but cannot degrade p53, and MDMX is apparently not present at a high enough concentration to effectively inhibit p53 activated death or arrest programs. If MDMX is deleted, MDM2 may either be too unstable to inactivate p53, or it may be less efficient at mediating p53 ubiquitination. As MDM2 and MDMX interact with each other, an implication of these studies is that a heterodimer of MDMX and MDM2 may be the most potent p53 inhibitor. An extension of these data is that conditions that interfere with MDMX binding to MDM2, that reduce the levels of either protein, or that affect MDM2 E3 ubiquitin ligase function could have profound effects on p53 regulation.

The impact of other ubiquitin ligases, de-ubiquitinating enzymes, and ARF on p53 and MDM2 stability and function

Pirh2 and COP1

Recent evidence suggests the existence of other ubiquitin ligases and de-ubiquitinating enzymes capable of modulating p53 and MDM2 levels and activities. Pirh2 is a RING domain protein that binds to the p53 DNA binding domain, and appears to induce p53 ubiquitination and degradation (Leng et al., 2003). Like MDM2, the Pirh2 gene is induced by p53. Pirh2 functions independently of MDM2, and is expressed in many tissues.

The human homolog of the Arabadopsis gene COP1 (constitutively photomorphogenic 1) has recently been identified as a p53 interacting RING finger protein able to ubiquitinate p53 (Dornan et al., 2004). Like Pirh2, it appears to be a p53-inducible gene, and when overexpressed, can reduce p53-dependent cell cycle arrest or apoptosis in cancer cell lines. Reducing COP1 levels by siRNA increases p53 levels, and sensitizes cells to damage induced activation of p53. COP1 overexpression reduced p53 levels in a co-transfection experiment using p53-/mdm2-null MEFs, suggesting that COP1 functions independently of MDM2. However, the physiologic significance of both COP1 and Pirh2 remains to be determined in light of the early embryonic lethality caused by MDM2 deletion. The failure of either COP1 or Pirh2 to rescue MDM2 deficiency is puzzling since cells lacking MDM2 should have activated p53 to induce high levels of these other putative p53 E3 ubiquitin ligases. It will be important to determine whether COP1 and Pirh2 regulate p53 in specific tissues, perhaps where MDM2/MDMX are limiting (Mendrysa et al., 2003).

HAUSP

Nearly 100 de-ubiquitinating proteins (DUBs) have been identified in the human genome (see Lima, 2003 for a review). A DUB that targets p53, MDM2 or MDMX, should affect p53 pathway regulation, but it is not easy to predict the effects. For example, a DUB directed against p53 should stabilize it, while one directed against MDM2 might have complex consequences depending on the ability of the de-ubiqutinated and presumably stabilized MDM2 to interact with and ubiquitinate p53. Work over the past several years has shown that there is at least one DUB, HAUSP (herpes associated ubiquitin-specific protease, also known as USP7), that targets p53 and MDM2, and that the consequences for p53 activation are indeed complex (Li et al., 2002; Lim et al., 2004; Wood, 2002).

HAUSP was identified as a p53-interacting protein (Li et al., 2002). It was initially proposed that HAUSP stabilized and activated p53 by removing ubiquitins from the p53 C-terminus. Importantly, although HAUSP may have many substrates, its overexpression only causes growth arrest in cells

expressing wild type p53, implying a p53-dependence to its growth inhibitory effects (Li et al., 2002).

Recent studies challenge the notion that HAUSP directly activates p53 via p53 de-ubiquitination. Two groups, one using siRNA (Li et al., 2004) and the other using homologous recombination in a tumor cell line to knock out HAUSP function (Cummings et al., 2004), showed that complete elimination of HAUSP caused p53 stabilization and growth arrest. This is contrary to expectation if the key HAUSP target is p53, as HAUSP elimination should leave p53 ubiquitinated, leading to its *destabilization.* However, the observed p53 activation by HAUSP knockdown can be explained if HAUSP's main target is MDM2. In this case, eliminating HAUSP should increase MDM2 ubiquitination, leading to its rapid degradation, and consequent activation of p53. In support of this, eliminating HAUSP caused accelerated MDM2 degradation and p53 stabilization (Li et al., 2004). Importantly, partial reduction of HAUSP produced the opposite result, in that p53 was partly destabilized (Li et al., 2004). It remains to be determined whether HAUSP activity or abundance can be regulated by growth conditions or stress and whether this affects MDM2 activity and p53 regulation *in vivo*.

ARF

Factors that affect the ability of MDM2 to ubiquitinate p53, or to control access of MDM2 to p53, should also contribute to p53 regulation. ARF, an alternative reading frame product of the INK4A locus (Kamijo et al., 1997; Sherr, 2001), binds to MDM2, p53, or both (Kamijo et al., 1998). Several mechanisms have been proposed for ARF mediated regulation of p53. ARF levels increase significantly in response to high level persistent signaling by Myc or oncogenically mutated Ras, or as MEFs and human fibroblasts become senescent (Dimri et al., 2000; Kamijo et al., 1997; Sherr, 2001). ARF is a nucleolar protein, and when induced to high levels in cells expressing oncogenes or nearing senescence, co-localizes with MDM2 in nucleoli (Weber et al., 1999). These observations led to the proposal that ARF may sequester MDM2 in the nucleolus, leading to MDM2 depletion from the nucleoplasm, and consequent activation of p53 (Weber et al., 1999). However, other studies show that ARF can activate p53 in the nucleoplasm (Llanos et al., 2001). This may be explained by the ability of ARF to bind to and inhibit the E3 ubiquitin ligase function of MDM2 (Honda and Yasuda, 1999), or by increasing the MDM2-mediated degradation of MDMX (Pan and Chen, 2003). In the latter case, decreasing MDMX levels should in turn destabilize MDM2, depleting the cell of both p53 negative regulators (Gu et al., 2002).

ARF contributes to efficient p53-dependent induction of apoptosis or cell cycle arrest in response to a subset of the signals that activate p53, but not in all tissues. Interestingly, one tumor in an ARF null animal contained mutated or inactivated p53, implying that both genes can collaborate in tumor progression and that p53 and ARF deficiencies are not functionally equivalent. For example, ARF contributes significantly to p53 activation induced by over-expressed Myc in B-cells, in p53-dependent senescence in MEFs growing *in vitro*, and in damage-induced responses of MEFs but not of other cell types such as intestinal epithelial cells (e.g., see Eischen et al., 1999; Kamijo et al., 1999b; Khan et al., 2000; Zindy et al., 1998). Also, ARF-null mice exhibit a different tumor spectrum and develop tumors with different latencies than p53 null mice (Kamijo et al., 1999a). Importantly, ARF does not appear to play a role in p53 dependent-apoptosis or tumor suppression in the mouse choroid plexus in which tumor progression is initiated by inactivating the retinoblastoma (Rb) tumor suppressor (Tolbert et al., 2002). This is noteworthy since Rb inactivation increases E2F1 activity in this system, and E2F1 has been shown to activate ARF in several *in vitro* systems (Bates et al., 1998; Dimri et al., 2000). Furthermore, in human fibroblasts, recent data show that decreasing ARF expression by siRNA enhances growth but does little to stimulate transformation induced by oncogenic ras, and that oncogenic ras still activates p53 when little if any ARF is present (Voorhoeve and Agami, 2004). Together, these data imply that ARF is an important, albeit species, cell and developmental stage specific modulator of p53 function, and that backup systems exist for activating p53 in tissues that do not express ARF.

p53 ACTIVATION BY POST-TRANSLATIONAL MODIFICATION

The current model

The data summarized above show that p53 function can be regulated by inhibitors including MDM2 and MDMX, by proteins that modulate the functions of MDM2/MDMX (e.g., ARF, HAUSP, etc.), and by proteins that may serve in both activating an inactivating capacities such as p300. However, p53 and MDM2 are also subject to rapid post-translational modifications on highly conserved serine and threonine residues by numerous protein kinases, and the functional impacts of these modifications are still uncertain (for detailed reviews and references, see (Appella and Anderson, 2001; Hay and Meek, 2000; Meek, 2002; Meek and Knippschild, 2003; Stewart and Pietenpol, 2001; Wahl and Carr, 2001). The clearest example for an essential role for phosphorylation in p53 activation comes from studies in *Drosophila*. DNA damage activates MNK, the *Drosophila* homolog of mammalian Chk2, to phosphorylate serine 4 in the p53 N-terminus (Brodsky et al., 2004; Peters et al., 2002). Mutation of MNK (Chk2), or of p53 serine 4

to alanine, prevented ionizing radiation from activating p53 or eliciting an apoptotic response (Brodsky et al., 2004; Peters et al., 2002). Importantly, p53 activation in flies occurred with a phosphorylation associated mobility shift, but was not accompanied by an increase in p53 abundance. These data indicate that phosphorylation does not activate fly p53 by changing its stability. This observation is consistent with the absence of a recognizable fly ortholog of MDM2. These data also provide compelling evidence that in *Drosophila*, an off-on switch for p53 activation is created by N-terminal phosphorylation by MNK (Chk2). However, the situation is not nearly so clear in mammalian cells.

Many studies in mammalian cells demonstrate that phosphorylation of multiple N-terminal serines in p53 is induced by DNA damage, as occurs for serine 4 in *Drosophila*. But in mammals, these phosphorylations seem to have more subtle effects on p53 function than was observed in flies. The first kinase identified for this role is one mutated in patients with ataxia telangiectasia (ATM), a disease associated with cancer predisposition, radiation sensitivity, chromosome abnormalities, and a failure to efficiently activate p53 in response to ionizing radiation (Banin et al., 1998; Canman et al., 1998; Kastan et al., 1992; Siliciano et al., 1997). The ATM kinase phosphorylates serine 15 in the p53 N-terminus, which is adjacent to the MDM2-binding domain (Shieh et al., 1997). An ATM-Rad3 related kinase (ATR) has also been reported to target serine 15 (Tibbetts et al., 1999). While serine 15 phosphorylation was initially proposed to prevent or reduce association of p53 with MDM2 (Shieh et al., 1997), other studies showed that serine 15 phosphorylation does not markedly affect MDM2 binding (Dumaz and Meek, 1999; Kane et al., 2000; Schon et al., 2002). Rather, serine 15 phosphorylation may enhance binding of the CBP co-activator (Dumaz and Meek, 1999; Lambert et al., 1998). Studies using phosphorylated peptides, *in vitro* binding, and transfection of relevant mutants suggested that phosphorylation of serine 20, probably in combination with threonine 18 and an N-terminal proline-rich region, mediate structural changes resulting in reduced affinity for MDM2 (Craig et al., 1999; Dumaz et al., 2001; Jabbur et al., 2002; Sakaguchi et al., 1998; Sakaguchi et al., 2000; Schon et al., 2002). However, other analyses of p53 mutants with one or more of the phosphorylation sites mutated have generated inconsistent results. For example, mutation of serine 20 alone, or in combination with five other N-terminal serines including serine 15 produced only a 50% reduction in the ability to induce apoptosis after transfection into H1299 cells (Unger et al., 1999a; Unger et al., 1999b). Two other studies showed that, in contrast to the work summarized above, mutation of N-terminal serine or threonine phosphorylation sites as well as others in the C-terminus, alone or in combination, had little effect on p53 stability or activation in cell culture

models (Ashcroft et al., 1999; Blattner et al., 1999). As the magnitude of the effects of p53 substitution mutations depends on the amount of transfected p53 relative to MDM2 expressed in the cells (Dumaz et al., 2001), the relevance of such analyses to control at normal physiologic levels remains uncertain.

A model has emerged emphasizing the importance of N-terminal phosphorylation in p53 activation (Sakaguchi et al., 1998). Phosphorylation of serines 15 and 20, and threonine 18 is proposed to induce a conformational change that prevents MDM2 from interacting with p53. This results in increased binding of p300/CBP, and presumably, the basal transcription machinery. As p300/CBP and the basal transcription machinery bind p53 in a region that partially overlaps that bound by MDM2, co-activator recruitment would compete for MDM2 binding (De Guzman et al., 2000; Lu and Levine, 1995; Thut et al., 1995; Xiao et al., 1994). Preventing MDM2 binding would increase p53 transcriptional output by increasing p53 abundance. p300/CBP binding to p53 should also lead p53 acetylation of C-terminal lysines; this could stabilize p53 by preventing MDM2-mediated ubiquitination of the same residues (Gu and Roeder, 1997; Nakamura et al., 2000; Rodriguez et al., 2000). p53 C-terminal acetylation has also been proposed to increase its ability to associate with chromatin, and to enable recruitment of another histone acetyl transferase, PCAF, that induces histone acetylation beyond that induced by p300/CBP (Barlev et al., 2001; Gu and Roeder, 1997; Liu et al., 1999).

While the model nicely integrates p53 N-terminal structure with the potential impact of phosphorylation on MDM2, p300/CBP and basal machinery binding, it has not been validated by analyses of the behavior of p53 phosphorylation site mutants. This could be explained in many ways, including the inability of the methods used to achieve physiologic levels of p53 or to generate the proper stoichiometric relationships between p53, MDM2 and MDMX. Therefore, a more rigorous test of the model is to use animal models, as described below.

Reconsidering p53 N-terminal phosphorylation

One prediction of the phoshporylation-acetylation cascade model for p53 activation is that binding of p300/CBP to the N-terminal transactivation domain is required for p53 to bind to chromatin. This prediction was tested using a mouse mutant in which residues leucine 25 and tryptophan 26 were changed to glutamine 25 and serine 26 (i.e., $p53^{QS}$). These residues are in the amphipathic alpha-helix that binds MDM2, p300/CBP, and the basal transcription machinery. The indicated substitutions prevent p53 acetylation

and transcriptional function (Chao et al., 2000b; Jimenez et al., 2000). However, $p53^{QS}$still binds as well as wild type p53 to p53 response elements in electrophoretic mobility shift experiments *in vitro* and to chromatin in MEFs (Chao et al., 2000b; Jimenez et al., 2000; M. Tang and G. Wahl, unpublished observations). This indicates that p300/CBP mediated p53 or chromatin acetylation is not required for p53 to bind the response elements of its target genes *in vivo*. These data are consistent with studies showing that stresses including leptomycin B treatment can activate p53 without inducing detectable C-terminal acetylation (Smart et al., 1999; Stommel and Wahl, 2004). Another implication is that C-terminal acetylation is not required for p53-mediated transcriptional regulation.

The regulatory importance of p53 N-terminal phosphorylation is being studied by making mutations of the conserved serine residues suggested by transfection experiments to be key contributors to p53 activation and stability. Mouse serines 18 and 23 (equivalent to human serines 15 and 20) have individually been mutated to alanine (S18A or S23A, respectively). The effects of the S18A mutation are important to determine as the phosphorylation-acetylation model predicts that C-terminal acetylation is dependent on prior phosphorylation of serine 18. Importantly, threonine 21 was not phosphorylated after DNA damage in the S18A mutant, which is consistent with the phosphorylation cascade initiating at serine 18. However, the *in vivo* data generated thus far do not point to an essential role for serine 18 phosphorylation in p53 activation in a majority of mouse tissues. p53 S18A in mouse embryonic stem cells, differentiated ES cells, or MEFs was present at nearly normal levels in unstressed cells, and was induced almost as well as wild type p53 in response to UV or ionizing radiation (Chao et al., 2003; Chao et al., 2000a; Sluss et al., 2004). While C-terminal acetylation was unaffected in differentiated ES cells, it appeared to be significantly reduced in MEFs (Chao et al., 2003; Chao et al., 2000a). S18A p53 exhibited equivalent binding to p53 response elements in chromatin using ChIP analysis, but some p53 target genes may be expressed at reduced levels (Chao et al., 2003). S18A MEFs arrested like wild type cells after ionizing radiation, but apoptosis in the thymus and spleen was reduced by 50% (Chao et al., 2003; Sluss et al., 2004). By contrast, S18A retinal cells exhibited only 20% of the wild type apoptotic response at 2Gy, but at 14 Gy appeared to undergo apoptosis at nearly wild type level (Borges et al., 2004). Of note, S18A was as effective at tumor suppression as wild type p53 (Sluss et al., 2004). Together, these *in vivo* analyses indicate that serine 18 phosporylation in mice is not essential for p53 activation in all tissues, and that inability to phosphorylate this residue does not compromise tumor suppression. Therefore, the phosphorylation-acetylation cascade may not be essential for p53 activation in mice. Alternatively, it may be important for p53 activation in only certain tissues, or

there may be additional mechanisms that contribute to p53 control that are independent of serine 18 and threonine 21 phosphorylation.

S23A mice have also been generated, and exhibited nearly wild type patterns of p53 activation and induction of apoptosis in ES cells or in thymocytes derived by RAG reconstitution (Wu et al., 2002). Therefore, S23A mutation did not compromise p53 stabilization or function in the tissues analyzed. This is not the result expected if phosphorylation at this position is required to reduce MDM2 binding after DNA damage (Craig et al., 1999; Shieh et al., 1999; Unger et al., 1999a). However, the only study performed thus far did not generate mice in which every cell contained the S23A mutation. Consequently, firm conclusions regarding the impact of S23A mutation on p53 function and tumor suppression will await analysis of mice expressing the mutation constitutionally so that tissue specific effects can be evaluated.

The observations made thus far in mouse models indicate that phosphorylation of serines 18 and 23 and threonine 21 is not essential for p53 activation. They also suggest that p53 can be activated by mechanisms that are independent of N-terminal phosphorylation. This interpretation is consistent with studies showing that merely disrupting p53-MDM2 interaction is sufficient to activate p53. For example, diffusible peptides that prevent MDM2 from binding to p53 induce cell cycle arrest or apoptosis without N-terminal phosphorylation (Bottger et al., 1997; Bottger et al., 1996; Garcia-Echeverria et al., 2000). In addition, a recent study described cis-imidazoline compounds (Nutlins) that mimic the p53 N-terminal alpha helical region that binds MDM2. Nanomolar concentrations of Nutlins activate p53 to induce either cell cycle arrest or apoptosis without measurable N-terminal modifications (Vassilev et al., 2004). The Nutlin effect is specific for MDM2 binding, as only Nutlin enantiomers that matched the p53 side chain conformation in the MDM2 binding site were active. These compounds effectively block MDM2-p53 interaction (Stommel and Wahl, 2004; Vassilev et al., 2004). These data demonstrate that preventing MDM2 from associating with p53 enables full p53 activation without stress-induced modifications.

Regulated MDM2 degradation is important for p53 activation

As a consequence of MDM2 being a p53 target gene, MDM2 transcripts and protein accumulate after p53 is activated (Barak et al., 1993; Michael and Oren, 2003; Perry et al., 1993; Wu et al., 1993). The activation of MDM2 by p53 establishes an auto-regulatory negative feedback loop to allow for finer tuning of the p53 response, and to reduce the chance of errant p53 activation (Lev Bar-Or et al., 2000; Wu et al., 1993). However, the activation of MDM2 by p53 also creates a problem in that p53 needs to be kept in an active form to initiate and maintain a stress response at the same time as MDM2 levels are

increasing. N-terminal p53 phosphorylations were supposed to allow for this by blocking MDM2-p53 interactions, but as discussed above, such modifications do not appear to be sufficient. Conversely, as the damage response wanes, mechanisms to turn off p53 will need to be restored, and it is currently unclear what these might entail. Below, we discuss data showing that another important response to DNA damage involves the accelerated degradation of MDM2. MDM2 destabilization is required for p53 activation to occur as MDM2 levels rise during a damage response. This process is triggered by damage-activated kinases, and requires the MDM2 RING domain (Stommel and Wahl, 2004). We therefore refer to this step in p53 activation as damage-activated MDM2 auto-degradation.

Efficient p53 activation requires the activity of damage-activated kinases such as ATM (Banin et al., 1998; Canman et al., 1998; Kastan et al., 1992; Siliciano et al., 1997). It has largely been assumed that p53 is the critical target for these modifications, but the nearly full activation of p53 N-terminal phosphorylation mutants suggests that this interpretation is incorrect. Rather, the data are more consistent with at least one additional substrate that is targeted by these kinases being involved in p53 activation. Importantly, MDM2 is phosphorylated by ATM with kinetics that are compatible with p53 activation (Khosravi et al., 1999; Maya et al., 2001). MDM2 phosphorylation was initially proposed to impede MDM2's ability to promote p53 export to the cytoplasm for degradation. However, as discussed above, p53 can be degraded efficiently in the nucleus, suggesting that MDM2 phosphorylation is required for a different step needed for p53 activation.

Our recent observations reveal that a critical step in p53 activation by DNA damage involves accelerated degradation of MDM2 (Stommel and Wahl, 2004). We first noticed that DNA damage decreased the stability of transfected MDM2, and confirmed this in normal human fibroblasts. We then observed a tight temporal correlation between the timing of accelerated MDM2 degradation and p53 activation. Within minutes after induction of DNA damage, ATM was activated, and p53 became phosphorylated on serine 15; but early after damage induction, serine 15 phosphorylated p53 was unstable, and was transcriptionally inactive. The instability of p53, along with its transcriptional inactivity, are consistent with p53 being able to interact with MDM2 at this time. Co-immune precipitation studies confirmed this idea, but we could only detect serine 15 phosphorylated p53 associated with MDM2 when we used proteasome inhibitors (for reasons discussed below). In contrast to the behavior of p53 soon after damage, between 1-2 hrs after damage induction, p53 became stabilized and p53 target genes were activated. Importantly, MDM2 was relatively stable at early times, and was significantly destabilized at 1-2hrs after damage induction, which correlates nicely with

p53 becoming stable and active. Later, as the damage response waned, p53 became unstable, transcriptionally inactive, and this correlated with re-stabilization of MDM2 (Stommel and Wahl, 2004).

We next asked whether MDM2 degradation is required for p53 activation. We reasoned that if accelerated MDM2 degradation is required for p53 activation, then preventing its degradation with proteasome inhibitors should prevent p53 from being activated. On the contrary, if phosphorylation of p53 and/or MDM2 could prevent their association, then proteasome inhibitors should not prevent p53 activation. Consistent with the model invoking MDM2 degradation in p53 activation, we observed that proteasome inhibitors prevented p53 mediated activation of p21 and MDM2. Importantly, under the conditions used, proteasome inhibitors did not prevent p53 from being phosphorylated on serine 15. One explanation of these results is that stabilizing MDM2 allows it to interact with p53 following DNA damage, resulting in p53 inactivation. This interpretation is consistent with the co-immune precipitation of serine 15 phosphorylated p53 with MDM2 in the presence of proteasome inhibitors. Importantly, adding an active Nutlin to prevent MDM2-p53 association prior to proteasome inhibition restored the ability of DNA damage to activate p53. This control shows that proteasome inhibitors do not block p53 activation by a non-specific mechanism. Rather, it is the MDM2 stabilization produced by the proteasome inhibitors, and the ability of the stabilized MDM2 to interact with serine 15 phosphorylated p53, that prevents p53 activation. These data imply that MDM2 destabilization during DNA damage contributes significantly to the inability of MDM2 to block p53 activation during a damage response.

Accelerated MDM2 degradation following DNA damage requires phosphorylation by damage activated kinases and is dependent on a functional MDM2 RING domain. Studies with ATM deficient cells, and phosphorylation site mutants of MDM2 suggest that more than one kinase, or more than one kinase target site in MDM2, may be involved in MDM2 destabilization (Stommel and Wahl, 2004). On the other hand, mutation of cysteine 464 in the MDM2 RING domain stabilized MDM2 and made it resistant to damage induced degradation. The mechanism by which damage-kinase mediated phosphorylation destabilizes MDM2 remains to be defined. One potential mechanism is that damage induced phosphorylation(s) enable recruitment of the E2 ubiquitin transferase via the RING domain. As MDMX prevents MDM2 auto-ubiquitination, and MDMX and MDM2 associate via their RING domains, it is also possible that destabilization results from phosphorylation induced dissociation of MDMX from MDM2. Interestingly, Yuan and colleagues recently found that DNA damage destabilized both MDM2 and MDMX (Kawai et al., 2003a). Therefore, it is also possible that DNA damage

could enhance MDM2 mediated ubiquitination of MDMX, which would accelerate both MDMX and MDM2 degradation.

Small changes in MDM2 abundance can affect p53 activation

The summary above makes it reasonable to expect that other factors that affect the abundance, stability, functionality or interaction of MDM2 and MDMX could set the threshold for p53 activation. Mitogenic levels of signaling from Raf and activation of NF-kB increase MDM2 levels sufficiently to make it more difficult to activate p53 by DNA damage (Ries et al., 2000; Tergaonkar et al., 2002). Similarly, activated AKT induces phosphorylation of MDM2 on at least two sites (S166, 186) resulting in nuclear accumulation (Gottlieb et al., 2002; Mayo and Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001a). While this has been reported to result from increased nuclear localization of MDM2 (Mayo and Donner, 2001), MDM2 is typically a predominantly nuclear protein, so the observed nuclear accumulation may instead result from increased stabilization of otherwise unstable nuclear MDM2. Stabilizing MDM2 should enable it to interact with and inhibit p53 more effectively.

Just as small increases in MDM2 levels can blunt p53 activation, small decreases in MDM2 levels make it easier to activate p53. For example, decreasing MDM2 levels by an average of 50% led to decreased body weight and reduced the size of multiple organs in p53+ but not p53- animals (Mendrysa et al., 2003). Decreasing MDM2 levels by at least 50% led to significantly increased sensitivity to ionizing radiation (Mendrysa et al., 2003). In another study, MDM2+/- mice exhibited greater resistance to Eu-Myc induced lymphomagenesis and to have greatly increased life spans due to drastic reduction of peripheral B cells by p53 (Alt et al., 2003). Peripheral and primary B cells from Eμ-Myc-MDM2+/- or MDM2+/- mice were far more sensitive to spontaneous apoptosis than those of wild type littermates, and loss of p53 rescued this sensitivity. Similarly, knockdown of MDM2 in zebra fish induced apoptosis, and arrested development at a very early stage (Langheinrich et al., 2002). These data provide compelling examples of how modest alterations in MDM2 abundance or stability produce profound effects on “spontaneous”, as well as oncogene and damage induced activation of p53.

A REVISED MODEL FOR p53 ACTIVATION

We propose the following revised model to account for p53 activation by DNA damage (see Figure 1). We envision two coordinated processes being involved. First, N-terminal phosphorylations in p53 may be important for it to cooperate with individual or preassembled components of the transcriptional regulatory machinery to send signals to RNA polymerase to convert it from

an inactive to an active state. Although p53 N-terminal phosphorylations were initially proposed to disrupt p53-MDM2 interactions, the data emerging from studies of mouse mutations, the inconsistencies in transfection analyses, and our immune-precipitation analyses, do not strongly support this idea. Also, in *Drosophila*, while N-terminal phosphorylation is critical for activation, flies appear to lack an MDM2 homolog.

The second step involves the ability of DNA damage to convert MDM2 into a more active E3 to accelerate the degradation of itself and MDMX. It is possible that accelerated degradation of MDM2 is sufficient since MDMX alone cannot efficiently inhibit p53-mediated biological responses. However, recent data indicate that MDMX degradation should further destabilize MDM2, which could contribute to reinforcing the activation process. Accelerated MDM2 degradation is a critical step, a inhibiting it prevents p53 activation, even when the p53 contains damage-associated modifications. This implies that effective recruitment of the transcriptional machinery to p53 responsive promoters, or of activating the transcriptional machinery once it is recruited, may require preventing MDM2 from interacting with p53.

It is tempting to speculate that complexes of p53, MDM2 and p300 might be chromatin-bound since all of these are (mainly) nuclear proteins, MDM2 binds to p53 best when p53 is tetrameric, and tetrameric p53 binds best to chromatin (Hainaut et al., 1994; Marston et al., 1995; McLure and Lee, 1998). Elimination of MDM2 from such complexes may enable rapid activation of p53 in response to stresses. This view is consistent with recent kinetic and ChIP analyses of p53 target gene activation showing that p53 and the basal transcription machinery are poised to function (Espinosa et al., 2003). Activating signals then induce phosphorylation of the C-terminal tail of the poised RNA polymerase to enable it to translocate along the DNA (Espinosa et al., 2003).

It is noteworthy that, like p53, other short-lived transcription factors including Myc, Hif1a, etc., have transactivation domains that overlap with the residues that mediate their destruction (Muratani and Tansey, 2003; Salghetti et al., 2000). Perhaps this organization evolved to allow for competition between the factors required for transcription factor activation with those needed for removal of the transcription factor from chromatin. We speculate that such an organization may have evolved to enable rapid proteolysis of chromatin bound transcription factors to prevent their errant activation; on the other hand, regulated degradation of an inhibitory E3 ubiquitin ligase enables its rapid removal from the complex to expedite transcriptional activation in response to the appropriate signal.

Advances in bioinformatics, molecular biology, biochemistry, and genetics are providing the bases for a detailed understanding of the circuitry that regulates p53 and tunes its output. This will provide a model for other pathways that process numerous signals to generate diverse responses. We are hopeful that advances in structural biology and chemistry will propel efforts to identify additional drugs capable of activating mutant p53 or wild type p53 in MDM2/MDMX over-expressing cells to enable selective activation of the p53 pathway. This class of targeted therapeutics should add significantly to the existing armamentarium to increase the success of cancer treatment in a broad range of neoplasms. This would be a great present to all who are still around to celebrate the 50th Anniversary of the discovery of p53!

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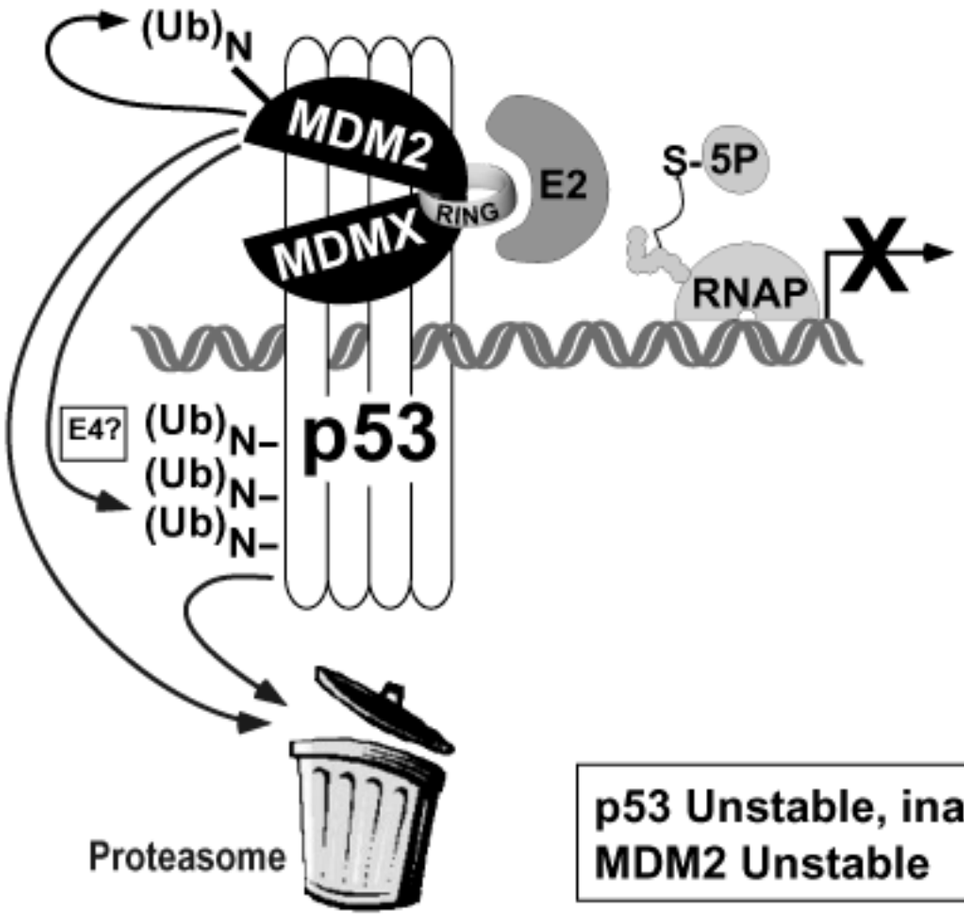
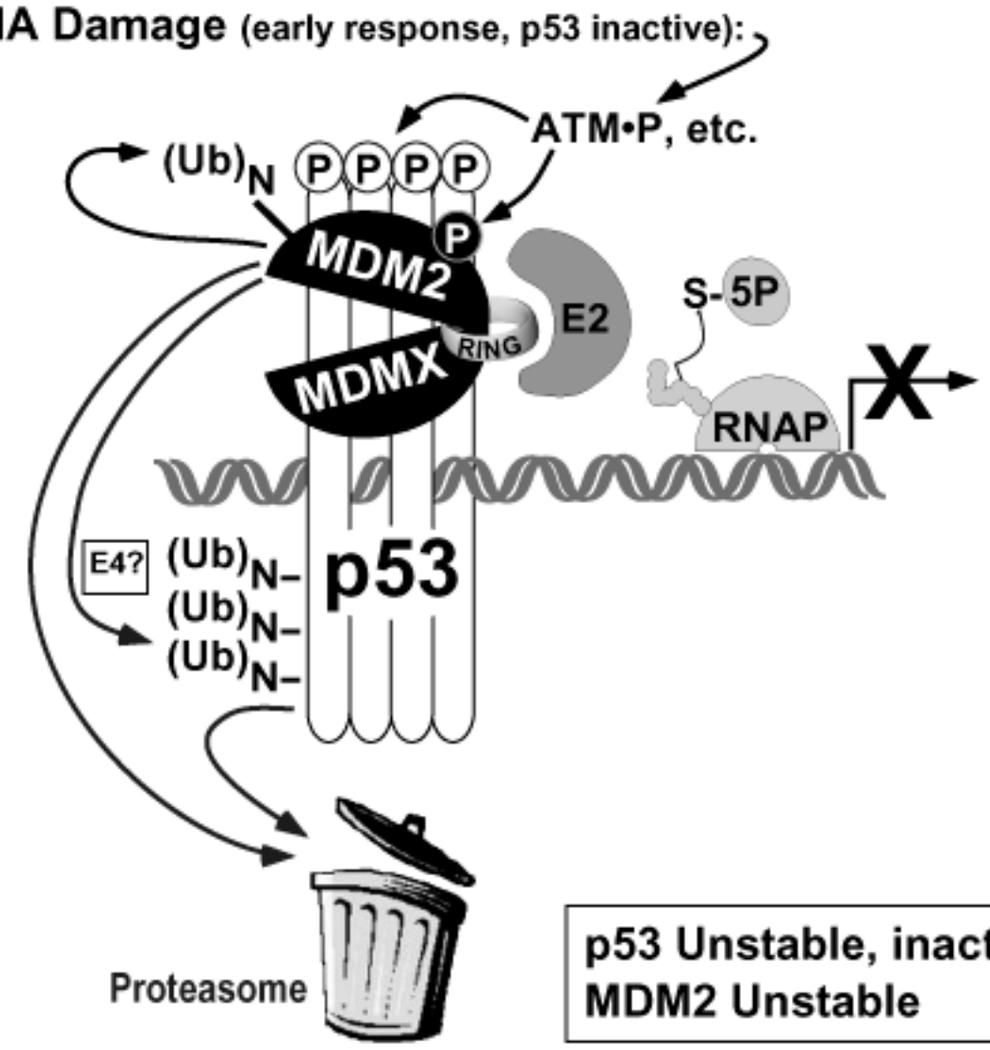
Undamaged: (p53 inactive)



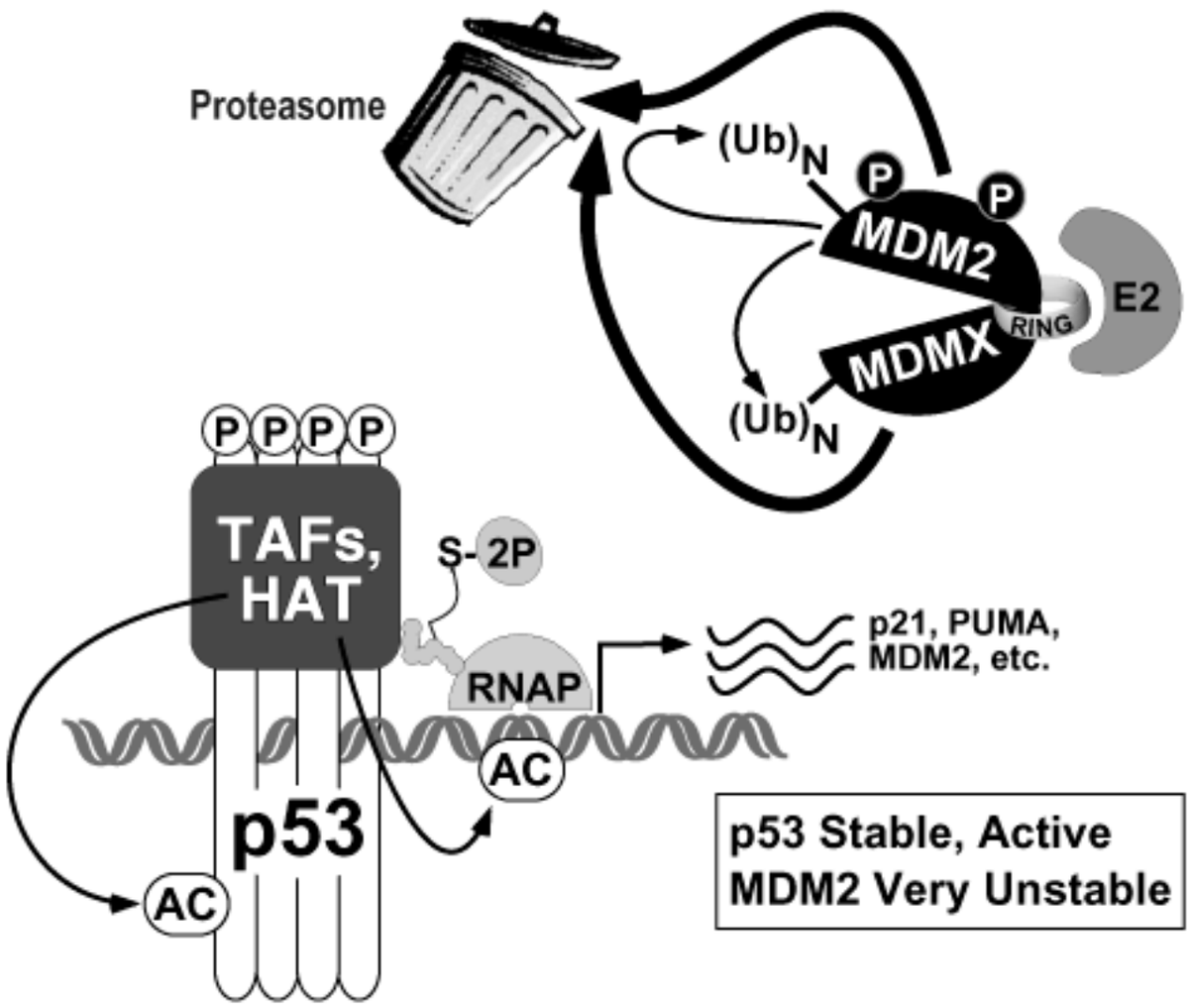
p53 Unstable, inactive
MDM2 Unstable

DNA Damage (early response, p53 inactive):



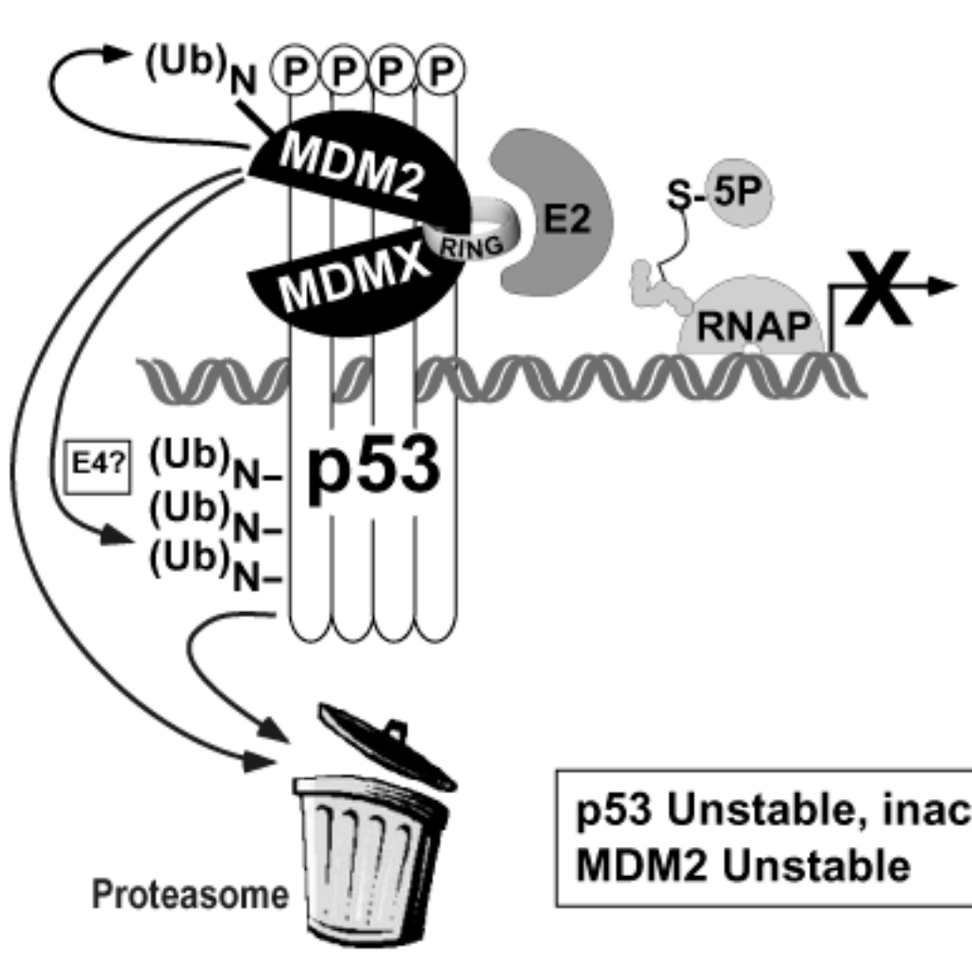
p53 Unstable, inactive
MDM2 Unstable

DNA Damage (peak response, p53 active):



p53 Stable, Active
MDM2 Very Unstable

DNA Damage (late, damage response attenuated, p53 inactive):



p53 Unstable, inactive
MDM2 Unstable