REVIEW

Limiting the power of p53 through the ubiquitin proteasome pathway

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The ubiquitin proteasome pathway is critical in restraining the activities of the p53 tumor suppressor. Numerous E3 and E4 ligases regulate p53 levels. Additionally, deubquitinating enzymes that modify p53 directly or indirectly also impact p53 function. When alterations of these proteins result in increased p53 activity, cells arrest in the cell cycle, senesce, or apoptose. On the other hand, alterations that result in decreased p53 levels yield tumor-prone phenotypes. This review focuses on the physiological relevance of these important regulators of p53 and their therapeutic implications.

The p53 tumor suppressor maintains genomic integrity by primarily functioning as a sequence-specific DNA-binding transcription factor (Vousden and Prives 2009). On sensing unfavorable cellular conditions, p53 transactivates downstream targets to induce apoptosis, cell cycle arrest, or senescence in affected cells and stops them from further propagating the damage (Riley et al. 2008). Therefore, it is not surprising to note that p53 activity is either impaired or attenuated in the majority of human cancers (Hernandez-Boussard et al. 1999; Olivier et al. 2009). While mutations in the *TP53* gene account for inactivation of its activity in >50% of human tumors (Soussi et al. 2006), attenuation of p53 activities by factors that decrease its levels is also a major contributor to undermining p53 function.

p53 is an unstable protein with an in vivo half-life of <20 min (Oren et al. 1981; Itahana et al. 2007; Pant et al. 2011). p53 protein turnover is manifested by a multitude of post-translational modifications that affect its stability and activity. Post-translational modifications of p53 include phosphorylation, ubiquitination, acetylation, methylation, sumoylation, neddylation, and glycosylation (Meek and Anderson 2009). Distinct p53 amino acids have been mapped that are targeted by modifying enzymes (Kruse and Gu 2008). Modification of these residues dictates p53 stability and impacts outcome. An important feature to note is that many of these modifications converge onto the same target residue or in the vicinity of the modified residue and can either cooperate or antagonize the effects of other modifications.

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Modification of p53 by ubiquitination and deubiquitination is an important reversible mechanism that effectively regulates its functions (for reviews, see Jain and Barton 2010; Brooks and Gu 2011; Love and Grossman 2012; Hock and Vousden 2014). Mono- or polyubiquitination of p53 by different E3 ligases regulates its nuclear export, mitochondrial translocation, protein stability, and transcriptional activity. Another set of enzymes called deubiquitinases (DUBs) can reverse these effects. Here, we focus on ubiquitination as a mechanism for regulating p53 stability and function and review current findings from in vivo models that evaluate the importance of the ubiquitin proteasome system in regulating p53.

Ubiquitination is critical for regulating p53

Ubiquitination is a post-translational modification that modifies target lysine amino acids on a protein and thus influences its function and turnover. It is conserved in eukaryotes, and analogous ubiquitin-like proteins have recently been reported in prokaryotes (Pearce et al. 2008). It is a multienzyme cascading process that involves three distinct sets of enzymes: E1-activating enzymes, E2-conjugating enzymes, and E3 ubiquitin ligases that work in quick succession to attach an evolutionarily conserved ubiquitin moiety of 76 amino acids to the lysine amino acid. As per recent estimates, the human genome contains only two E1-encoding genes, ~40 E2-encoding genes, and >600 different E3 ligases (Li et al. 2008). E1 enzymes initiate the ubiquitin reaction by ATP-dependent activation of ubiquitin and tether it to an E2. The E3 ligases ascertain the specificity of the substrate and facilitate the transfer of this activated complex to the target protein (David et al. 2011). Ubiquitin chains established by sequential K48 linkage (polyubiquitination) lead to protein degradation via 26S proteasome, while K63-linked ubiquitin chains regulate signaling (Thrower et al. 2000). E3 ligases can also simply mono-ubiquitinate lysine residues of a protein, an event that signals further regulation of the protein (Hicke and Dunn

2003). E3 ligases are broadly classified into two groups based on their catalytic domain: (1) RING (really interesting new gene) domain E3 ligases and (2) HECT (homologous to E6-AP C terminus) domain E3 ligases. E4 ligases, also called ubiquitin chain elongating factors, represent a new class of ubiquitin enzymes that mediate the elongation of the ubiquitin chain that was previously established by the E3 ligases (Fig. 1; Koegl et al. 1999). For a more detailed overview of the ubiquitin system, please refer to Deshaies and Joazeiro (2009), Lipkowitz and Weissman (2011), and Varshavsky (2012).

An array of E3 ligases belonging to either the RING or HECT subgroups has been identified that targets multiple lysines on p53 for ubiquitination.

The Mdm2 E3 ligase

Mdm2 is an E3 ubiquitin ligase of the RING finger class that regulates p53 stability and activity (Haupt et al. 1997; Honda et al. 1997; Kubbutat et al. 1997; Marine and Lozano 2009). In mice, deletion of *Mdm2* leads to p53-dependent cell death phenotypes (Lozano 2010), clearly designating p53 as an Mdm2 substrate. The E3 ligase activity of Mdm2 is primarily encoded by the RING domain (Fang et al. 2000). However, recent studies have also implicated the extreme C-terminal amino acids of Mdm2 in E3 ligase function (Uldrijan et al. 2007). Mutations of cysteine residues in human MDM2 (C447, C462, or C475) that are critical for the structure of the RING domain (Sharp et al. 1999; Argentini et al. 2000; Fang et al. 2000) or changes in the C-terminal tail length by either deletion of five amino acids or extension of five residues (by bypassing the stop codon) substantially inhibit its E3 ligase activity (Poyurovsky et al. 2010; Dolezelova et al. 2012). Another important feature of the Mdm2 RING domain is that it interacts with an Mdm2-related protein, Mdm4 (Sharp et al. 1999; Tanimura et al. 1999). Disruption of Mdm2–Mdm4 interaction results in an embryo-lethal phenotype that is also p53-dependent (Huang et al. 2011; Pant et al. 2011). Other mutant mice with deletion of the entire RING domain or with a point mutation (C462A) in the Mdm2 RING domain, both of which disrupt the E3 ligase activity and interactions with Mdm4, are also p53-dependent embryo-lethal (Steinman and Jones 2002; Itahana et al. 2007). To date, it has not been possible to genetically distinguish RING ligase activity from Mdm4 binding. Physiological disruption of the interaction between p53 and Mdm2/Mdm4 also occurs upon post-translational modifications and impacts p53 ubiquitination (Prives and Hall 1999; Ito et al. 2002; Li et al. 2002; Chao et al. 2003, 2006; Xirodimas et al. 2004; Wang et al. 2009; Gannon et al. 2012). Thus, the activity of the Mdm2 E3 ligase toward p53 is regulated by multiple parameters: the RING domain, the C terminus, and interactions with Mdm4.

Variations in Mdm2 levels are also important determinants of p53 function and impact tumor phenotypes (Eischen and Lozano 2014). For example, low Mdm2 levels induce monoubiquitination and nuclear export of p53, while higher levels promote its polyubiquitination and degradation (Li et al. 2003). Single-nucleotide polymorphisms (SNPs) in *Mdm2* have been discovered that regulate Mdm2 levels and impact p53 activity, contributing to tumor risk (Bond et al. 2004; Post et al. 2010; Knappskog et al. 2011). These variations affect basal levels of Mdm2 and may alter p53 mono- and polyubiquitination. Transgenic mice that overexpress Mdm2 are also tumor-prone (Jones et al. 1998). Likewise, overexpression or amplification of *Mdm2* is observed in multiple human tumors, including sarcomas, gliomas, and leukemias,

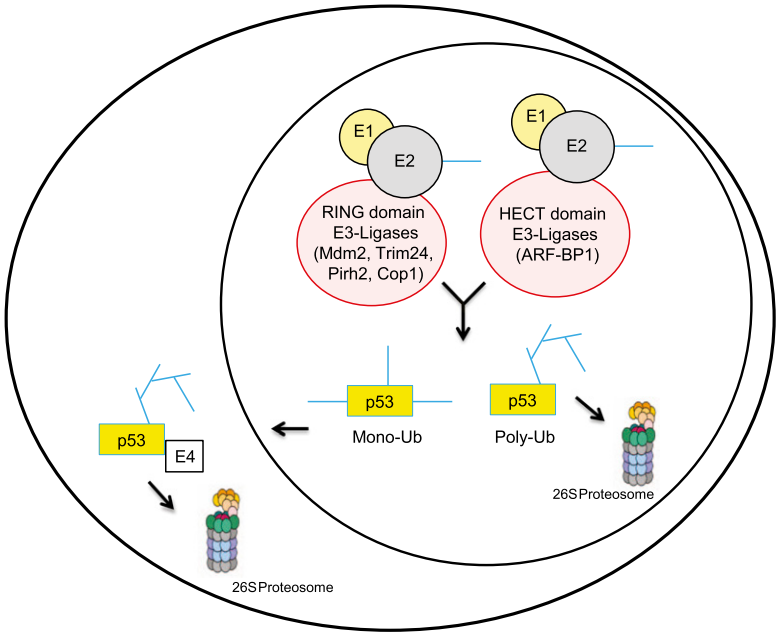

Figure 1. A simplistic overview of p53 ubiquitination.

many of which retain wild-type p53 (Bueso-Ramos et al. 1993; Oliner et al. 1993; Jones et al. 1998).

Incidentally, in vitro experiments suggest that Mdm2 is also an ubiquitin ligase for itself, and this autoubiquitination is impeded by its interaction with Mdm4 (Fang et al. 2000). However, this was not confirmed in *Mdm4ΔRING* mice, which are defective in Mdm2–Mdm4 interaction (Pant et al. 2011). Furthermore, the ubiquitination profile of Mdm2 was also not altered in *Mdm2C462A* mice, which lack both E3 ligase activity and interactions with Mdm4 (Itahana et al. 2007). Another mechanism of altering Mdm2 levels is through its interaction with Arf. Arf induces p53 levels by sequestering Mdm2 into the nucleolus and also by binding to Arf-BP1 (Arf-binding protein 1), another E3 ligase for p53 (Weber et al. 1999; see below). Changes to the p53 ubiquitination profile in $Arf^{-/-}$ mice were not examined, although the mice are tumor-prone (Kamijo et al. 1999).

Mdm2 targets six key lysine amino acids on p53 (K370, K372, K373, K381, K382, and K386) for ubiquitination (Rodriguez et al. 2000). In vitro studies show that mutation of these lysine residues decrease p53 ubiquitination, increasing its activity. However, knock-in mice with lysine-to-arginine mutations that prevent ubiquitination but do not modify charge at these six residues (*p53-6KR*) do not exhibit significantly increased p53 levels (Feng et al. 2005). Another knock-in mouse (*p53-7KR*) in which an additional lysine residue (that is present only in the mouse) was changed also showed p53 levels that were comparable with wild-type mice (Krummel et al. 2005). Notably, Mdm2 can still bind to and degrade a truncated form of p53 that is missing these C-terminal lysine residues (Kubbutat et al. 1998; Poyurovsky et al. 2010). This highlights two important points: (1) Additional lysines on p53 may regulate p53 stability via ubiquitination, and (2) the Mdm2 E3-ligase is promiscuous, as it can target other lysines in the absence of its preferred residues. In fact, it has been shown that several lysines in the p53 DNA-binding domain are also critical for its ubiquitination (Chan et al. 2006). However, this has not been confirmed in vivo. Another possibility is that other E3 ligases exist that cooperate to target p53 for degradation.

The p53 protein is stabilized after DNA damage and must be destabilized for cell survival. Mdm2 also regulates this transition. Mdm2 is itself a transcriptional target of p53. The P2 promoter of *Mdm2* has two distinct p53-binding sites by which the p53 induced by DNA damage binds and activates *Mdm2* transcription (Barak et al. 1993; Wu et al. 1993; Saucedo et al. 1998). This results in a feedback loop between p53 and Mdm2 in which p53 induces expression of its inhibitor after the cell recovers from stress. The long-held paradigm in the field suggested that the feedback loop is the primary mode through which p53 autoregulates its levels and activity after DNA damage. We recently tested this paradigm in a mouse model in which the feedback loop was disrupted due to mutations in the p53-binding sites in the *Mdm2* P2 promoter. While mice lacking the p53-binding sequences in the P2 promoter are normal, they exhibit hematopoietic defects and die after exposure to low-dose ionizing radiation (Pant et al. 2013; Pant and Lozano 2014). Nonetheless, the pattern of p53 stabilization and destabilization remains nearly identical between wild-type and mutant mouse cells/tissues after ultraviolet-induced or ionizing radiation-induced DNA damage. Although p53 stability under other stress conditions remains to be examined, these data suggest that other factors may be involved in regulation of p53 stability. These data also make another important point: Stabilization of p53 does not necessarily equate to increased activity.

Mdm2 binds the N-terminal transactivation domain of p53, which is intact in the p53 hot-spot mutations found in human cancers. This observation suggested that Mdm2 might also regulate stability of mutant p53. The fact that missense mutations in the DNA-binding domain render p53 incapable of transcriptional activity provides an ideal model system to examine mutant p53 as a substrate for Mdm2 E3 ligase function. *p53R172H* homozygous mutant mice could rescue the *Mdm2*-null phenotype (Terzian et al. 2008). Moreover, loss of *Mdm2* leads to increased levels of mutant p53R172H protein in many, but not all, cells. We also noted that the p53R172P mutant protein degrades in an Mdm2-dependent manner in mouse embryonic fibroblasts (MEFs) (Liu et al. 2007). These data suggest that mutant p53 is also a substrate of Mdm2. Recently, CHIP (C terminus of HSP70-interacting protein) has been identified as another E3 ligase that targets mutant p53 (Lukashchuk and Vousden 2007). The chain of events leading to degradation of mutant p53 likely follows the same pathway as that of wild-type p53 (Suh et al. 2011). Since p53 missense mutations lack p53 activity, they may serve as a readout to study other E3 ligases that might regulate p53 degradation.

Mdm2 is not alone

p53 degradation has been observed in the absence of Mdm2. Specifically, a conditional mouse model with a p53-ER fusion that can be toggled between inactive and active states shows that restored wild-type p53 on an *Mdm2*-null background eventually degrades, although the rate of decay is slower (Ringshausen et al. 2006). More evidence in support of this idea comes from the fact that p53R172H degradation still takes place, albeit at a slower rate, in an *Mdm2*-null background (Terzian et al. 2008). The delay in p53 degradation in Mdm2-deficient mice points to the involvement of other enzymes in the ubiquitination and degradation process of p53 during homeostatic conditions.

Even in response to DNA damage, other E3 ubiquitin ligases in addition to Mdm2 may function to regulate p53 levels. Using the mouse model in which the p53–Mdm2 feedback loop is disrupted, we observed that DNA damage-stabilized p53 degraded just like in wild-type tissues, but the rate of degradation was slightly delayed. This suggests that other proteins cooperate with Mdm2 in degradation of p53 after stress (Pant et al. 2013; Pant and Lozano 2014). Unfortunately, ubiquitination was not assayed in tissues from these mice due to technical challenges. Together, these data convincingly implicate other E3 ligases in the regulation of p53 stability.

Pirh2 (p53-induced protein with a RING-H2 domain) is another E3 ligase that targets K101, K164, K292, K305, K357, K382, and K386 and promotes p53 ubiquitination (Leng et al. 2003). Overexpression of Pirh2 in cells leads to decreased p53-mediated apoptosis and cell cycle arrest (Leng et al. 2003). However, *Pirh2*-null mice are viable and develop normally, suggesting that Pirh2-mediated inhibition of p53 is not essential, as is Mdm2 during early development (Hakem et al. 2011). Under homeostatic conditions, *Pirh2*-null tissues display only a mild increase in p53 levels and no significant increase in p53 activity. However, after radiation exposure, splenocytes and thymi derived from *Pirh2*$^{-/-}$ mice exhibit increased levels of p53 and enhanced p53 transcriptional activity, suggesting a role for Pirh2 in DNA damage response (Hakem et al. 2011). Unfortunately, in this study, the investigators did not directly examine p53 levels in irradiated mouse tissues without exposing them to culture conditions, a stress stimulus in itself. Also, unlike *Mdm2*$^{+/-}$ mice, which are radiosensitive (death after sublethal irradiation) (Terzian et al. 2007), overall radiosensitivity of the *Pirh2*-null mice was not evaluated. These data imply that Pirh2 is a rather weak p53-stabilizing/activating agent in vivo. However, if overexpressed, Pirh2 may have a greater impact on p53 inhibition. Increased PIRH2 levels are observed in lung cancer and prostate cancer and correlate with disease progression, expanding the possibility that, when overexpressed, it may have a more dominant role in inhibition of p53 (Duan et al. 2004; Logan et al. 2006).

Trim24 belongs to the ever longer-growing list of Trim proteins that possess E3 ligase activity. Trim24 was identified as a p53-binding partner in screens using TAP-tagged p53 as the bait protein (Allton et al. 2009). Biochemical analysis revealed that Trim24 is a RING domain E3 ligase for p53. Depletion of Trim24 in various cell lines results in a corresponding increase in p53 levels. To understand the biological relevance of Trim24 in p53 regulation, Allton et al. (2009) deleted *Bonus*, the homolog of Trim24 in *Drosophila*. *Trim24* loss results in enhanced apoptosis in the *Drosophila* wing imaginal discs, an in vivo phenotype that is rescued by p53 RNAi, implicating Trim24 in p53 inhibition. The finding that *Trim24*$^{-/-}$ mice are viable questions the in vivo E3 ligase capabilities of this protein on p53, at least during early development (Khetchoumian et al. 2007). Possibly, Trim24 has a tissue/stress-specific role in p53 ubiquitination, or functional redundancy among multiple Trim protein family members masks the effect of *Trim24* loss in vertebrates. But again, increased levels of Trim24 likely impact p53 functions. For example, Trim24 overexpression in immortalized human mammary epithelial cells reduces p53 levels dramatically (Pathiraja et al. 2014). TRIM24 is overexpressed in breast cancer and other human tumors (Tsai et al. 2010; Chambon et al. 2011). A recent study provides evidence that Trim24 prefers phosphorylated p53 for targeting (Jain et al. 2014). This would imply that Trim24 is responsible for degrading active p53.

In vitro binding assays and transfection studies in human cell lines also identified Cop1 (constitutive photomorphogenesis protein 1) as a putative E3 ubiquitin ligase for p53. Two groups independently generated *Cop1* hypomorphic and knockout mice (Migliorini et al. 2011; Vitari et al. 2011). Surprisingly, these mice die prematurely during embryonic development at 15.5 d postcoitum (dpc) due to cardiovascular defects. However, unlike *Mdm2* loss, this is not a p53-dependent phenotype. Moreover, *Cop1*-deficient mouse tissues/cells do not exhibit increased p53 levels or activity (Migliorini et al. 2011). Even after exposure to DNA-damaging agents that stabilize p53, such as ultraviolet light, doxorubicin, or Nutlin 3a, there was no difference in p53 stability in wild-type versus *Cop1*$^{-/-}$ MEFs. *Cop1*-deficient MEFs proliferate normally in culture and do not exhibit p53-dependent growth arrest phenotypes. Possibly, Mdm2 or other E3 ubiquitin ligases mask or compensate for Cop1 activity on p53. Also, the Cop1–p53 interaction could be important in certain contexts, which are obfuscated because of the embryo-lethal phenotype. On a slightly different note, perhaps when overexpressed, as in tissue culture or in tumors, Cop1 can target p53 for proteasomal degradation. High COP1 expression is reported in ~80% of human breast adenocarcinomas and 45% of ovarian adenocarcinomas (Dornan et al. 2004). COP1 is also expressed in hepatocellular carcinomas and pancreatic cancer (Lee et al. 2010; Su et al. 2011). Unfortunately, the mutation status and levels of p53 were not examined in tumors with high Cop1 levels. This puts Pirh2, Trim24, and Cop1 in a unique class of RING E3 ligases that may regulate p53 only under special circumstances. However, upon overexpression, as occurs in human cancers, these proteins could be effective p53 inhibitors. A comparison of p53 status (mutations and loss of heterozygosity) in tumor samples with or without high levels of these three ligases is critical for understanding the in vivo significance and impact on inhibition of p53 activity.

In contrast to the above examples of RING domain E3 ligases, Arf-BP1 is an HECT domain containing E3 ligase that can ubiquitinate and degrade p53 in cells. Like Mdm2, Arf-BP1 directly binds and ubiquitinates p53, and this activity is strongly inhibited by ARF, an Mdm2-binding protein that sequesters Mdm2 away from p53 (Weber et al. 1999). Genetic ablation of *Arf-BP1* in mice leads to early embryonic death ~14.5 dpc (Kon et al. 2012). This is accompanied by slight increases in p53 levels and a corresponding increase in apoptosis in some tissues, but crosses with *p53*-null mice were not reported. More localized deletion of *Arf-BP1* in pancreatic β cells results in an age-dependent diabetic phenotype due to a p53-dependent decrease in the β-cell population. Thus, Arf-BP1 appears to inhibit p53 activity in a limited fashion. ARF-BP1 is also overexpressed in a range of primary tumors and may have a greater effect on p53 in this context (Adhikary et al. 2005, Yoon et al. 2005).

Many other novel proteins have been described for p53 ubiquitination and degradation. However, thorough genetic evaluation in support of these claims is still lacking. For example, Topors, a RING ligase, mediates polyubiquitination of p53 and decreases its expression levels (Rajendra et al. 2004). Homozygous mice that lack *Topors* exhibit a significantly reduced life span, with several mice

showing signs of premature aging, including kyphosis, and increased incidence of multiple tumors (Marshall et al. 2010). Increased p53 activity has been implicated in aging defects (Tyner et al. 2002). Whether the phenotype in *Topors*-null mice is due to extended stability/activity of p53 has not been examined. CARP1 and CARP2 also polyubiquitinate p53 and inhibit p53 transactivation. CARP proteins promote degradation of unphosphorylated and S-20 phosphorylated p53 in cells (Yang et al. 2007). *CARP2*-deficient mice develop normally (Ahmed et al. 2009). Possibly, the structural homolog CARP1 can compensate for CARP2 loss. A double-mutant mouse with simultaneous deletion of both CARP proteins could be used to test this hypothesis. Synoviolin, an endoplasmic reticulum (ER) protein, sequesters p53 in the ER for degradation (Yamasaki et al. 2007). Synoviolin can polyubiquitinate p53 both in vitro and in vivo. Knockdown of Synoviolin increases p53 levels and p53 transcriptional activity promoting cell cycle arrest. *Synoviolin* deletion in mice causes embryonic death ~13.5 dpc, with apparent hypocellularity and aberrant apoptosis in fetal livers (Yagishita et al. 2005). Definitive erythropoiesis is also affected in a non-cell-autonomous manner. The role of p53 in these mice has not been evaluated. CHIP, an E3 ligase that also functions as an E4 in certain contexts, participates in p53 degradation by recruiting other proteins, such as HSP70 and HSP90 chaperone complexes (Dai et al. 2003; Esser et al. 2005). $CHIP^{-/-}$ mice have atrophied thymi and display a reduced life span and accelerated aging phenotypes accompanied by accelerated cellular senescence and increased indication of oxidative stress (Min et al. 2008). Again, the role of p53 stability/activity in manifestation of this phenotype has not been examined. The in vivo role of these E3 ligases is summarized in Table 1.

Genetic studies clearly indicate that these E3 ligases do not appear to inhibit p53 as dramatically as Mdm2. This could mean that they are either redundant, involved subsequent to Mdm2 monoubiquitination of p53, or only important in overexpressed systems such as certain tumors or under specific stress conditions. To address these concerns, more thorough in vivo investigations in multiple tissue types, under different stress and developmental conditions, and possibly in a limiting Mdm2 background are required. Conditional deletion or overexpression mouse models would also be more informative.

E4 ligases in p53 ubiquitination

The existence of E4 ligases represents a relatively new concept in the field. E4 ligases function to elongate ubiquitin chains previously set up by E3 ligases. The histone acetyltransferase (HAT) p300 and its paralog, CBP (CREB-binding protein), are the first set of proteins identified to possess an intrinsic E4 activity toward p53 in vitro and in cell culture (Grossman et al. 1998). These proteins are present in both nuclear and cytoplasmic compartments of a cell. While the proteins function as HATs in the nucleus, their E4 activity is exclusively observed in the cytoplasm (Grossman et al. 2003). Under normal conditions, both p300 and CBP promote polyubiquitination of p53 that is already monoubiquitinated by Mdm2 and in the cytoplasm. Mice lacking *p300* are early embryo-lethal by 11.5 dpc due to severe central nervous system and heart abnormalities (Yao et al. 1998). Due to the essential role of these proteins as HATs and their nuclear–cytoplasmic localization, it is difficult to confirm their E4 role in vivo. Identification of point mutations in p300 and CBP that can separate their HAT and E4 functions would be informative.

Other E4 ligases function primarily by enhancing the processivity of E3 ligases. UBE4B is an E3 ligase that can also function as an E4. It possesses a noncanonical U-box domain that interacts with both Mdm2 and p53 in vitro and in cells (Hoppe et al. 2000). UBE4B extends the ubiquitin chains placed by Mdm2 on p53. Thus, the UBE4B E4 ligase activity on p53 is dependent on Mdm2 (Wu et al. 2011). Deletion of UBE4B in vivo leads to early embryonic death due to induction of apoptosis in the heart, an organ where it is exclusively expressed during this developmental stage (Kaneko-Oshikawa et al. 2005). Whether this apoptosis is credited to p53 stabilization is not clear. MEFs derived from $UBE4B^{-/-}$ mice exhibit greatly reduced p53 polyubiquitination activity and correspondingly elevated basal p53 levels.

Gankyrin is an ankyrin repeat-containing protein that associates with the ATPase subunit of 26S proteasome (Higashitsuji et al. 2005). Gankyrin increases the interaction of ubiquitinated p53 and Mdm2 with the proteasome. Overexpression of Gankyrin in cells increases the ratio of polyubiquitinated versus monoubiquitinated p53. Overexpression of Gankyrin is reported in hepatocellular carcinoma, colorectal cancer, pancreatic cancer, esophageal squamous cell carcinoma, and breast cancer (Zheng et al. 2014). Gankyrin is located on the X chromosome and has not been deleted in model systems to analyze its functions.

Yin-Yang 1 (YY1) is another E4 that enhances p53 polyubiquitination through enhancing the p53–Mdm2 interaction (Sui et al. 2004). YY1 binds p300 and promotes p300 mediated polyubiquitination of p53 (Lee et al. 1995). *YY1* knockout mice have altered heterochromatin integrity and exhibit impaired spermatogenesis (Wu et al. 2009). The role of p53 in the phenotype has not been investigated. YY1 is overexpressed in human breast cancer, prostate carcinoma, acute myeloid leukemia, osteosarcoma, cervical cancer, brain cancer, ovarian cancer, large B-cell and follicular lymphoma, and colon cancer (for review, see Sui 2009).

It is still not clear whether E4s are absolutely necessary for elongation of ubiquitin chains set up by E3 ligases. Similarly, whether E4s are E3-specific is not clear. As with E3 ligases, increased expression of E4 ligases may impact tumor development and synergize with E3 activities to promote tumorigenesis.

Mdm4, an E4 ligase?

Mdm4 is a structural homolog of Mdm2 and can similarly bind and inhibit p53 activity. However, the RING domain of Mdm4 does not possess an analogous E3 ligase activity

Table 1. *Summary of phenotypes in animal models after deletion of ubiquitin enzymes*

Enzyme	Class, type	Effect on p53	In vivo model, phenotype	References
Mdm2	E3, RING	Monoubiquitination, nuclear export, polyubiquitination, degradation	Mouse, embryo-lethal 3.5 dpc, rescued by p53 deletion; conditional deletion in most radiosensitive tissues also lethal	Jones et al. 1995; Montes de Oca Luna et al. 1995; Xiong et al. 2006; Francoz et al. 2006; Ringshausen et al. 2006; Zhang et al. 2014
Pirh2	E3, RING	Degradation	Mouse, viable, enhanced radio response	Hakem et al. 2011
Trim24	E3, RING	Degradation	*Drosophila*, increased apoptosis in wing discs rescued by p53 RNAi, mouse, viable, tumor-prone	Khetchoumian et al. 2007
Cop1	E3, RING	Degradation	Mouse, embryo-lethal, no rescue by p53	Migliorini et al. 2011; Vitari et al. 2011
ARF-BP1	E3, HECT	Degradation	Mouse, lethal 14.5 dpc, no rescue by p53; conditional deletion in pancreatic β cells results in age-dependent diabetes, partially recovered by loss of p53	Kon et al. 2012; Adhikary et al. 2005; Yoon et al. 2005
Topors	E3, RING	Degradation	Mouse, reduced life span, aging phenotypes	Marshall et al. 2010
Carp1/Carp2	E3, RING	Degradation	Mouse, normal	Ahmed et al. 2009
Synoviolin	E3, RING	Degradation	Mouse, embryo-lethal 13.5 dpc, hypocellularity and apoptosis of liver	Yagishita et al. 2005
CHIP	E3, U box	Degradation	Mouse, aging phenotypes	Dai et al. 2003; Min et al. 2008
MSL2	E3	Nuclear export	Chicken cells, increased DNA damage response	Lai et al. 2013
P300/CBP	E4	Degradation	Mouse, lethal, 11.5 dpc, central nervous system and heart abnormalities	Yao et al. 1998
UBE4B	E4	Degradation	Mouse, embryo-lethal, apoptosis in heart	Kaneko-Oshikawa et al. 2005
Gankyrin	E4	Degradation	No in vivo model	Higashitsuji et al. 2005
Yin Yang 1	E4	Degradation	Mouse, impaired spermatogenesis	Wu et al. 2009
Hausp	DUB	Degradation	Mouse, lethal, 6.5–7.5 dpc, severe reduction of cell proliferation	Kon et al. 2010
USP10	DUB	Stabilization	Mouse, lethal, reactive oxygen species regulation defects	Takahashi et al. 2013
USP42	DUB	Stabilization	No in vivo model	Hock et al. 2011

(Shvarts et al. 1996; Stad et al. 2001). Still, Mdm4's role in p53 regulation is undeniable. *Mdm4* loss leads to a p53-dependent embryo-lethal phenotype (Parant et al. 2001; Migliorini et al. 2002). While Mdm2 forms homodimers, it forms heterodimers with Mdm4 more efficiently (Cheng et al. 2011). At physiological levels, Mdm2 homodimers predominantly monoubiquitinate p53 and promote its nuclear export. Mdm4 is required to potentiate the E3 ligase function of Mdm2 and eventual p53 degradation (Linares et al. 2003). Mdm2 homodimers are formed at higher protein levels, and these dimers can polyubiquitinate p53. Perhaps this is the reason that Mdm2 transgenic mice can rescue lethality of *Mdm4*-null mice (Steinman et al. 2005). Moreover, inactivation of Mdm2, but not Mdm4, leads to increased p53 levels in mice (Francoz et al. 2006; Xiong et al. 2006; Barboza et al. 2008). Since Mdm4 does not possess an intrinsic E3 activity, we argue that it could instead act as a cofactor or E4 to convert Mdm2 monoubiquitination marks to polyubiquitination. This hypothesis can be easily tested in vitro by examining changes to the ubiquitination profile of monoubiquitinated p53 after addition of Mdm4.

There is no evidence that Mdm4 without Mdm2 can degrade p53 in vivo. In fact, no change in the stability of a temperature-sensitive mutant p53 was observed upon deletion of Mdm4 (Barboza et al. 2008). Similarly, no noticeable difference in p53 ubiquitination pattern or p53 stability was observed in two other Mdm4 mutant mice, *Mdm4ΔRING* or *MdmX-3SA* (Wang et al. 2009; Pant et al. 2011). However, it is possible that the presence of Mdm2 masked the effect on p53 stability. The role of Mdm4 in p53 degradation may be addressed either in cells/tissues from conditional mice in which p53 can be restored in an *Mdm2/Mdm4* double-null background or by comparing the degradation profile of p53R172H in $Mdm2^{-/-}p53^{H/H}$, $Mdm4^{-/-}p53^{H/H}$, and $Mdm2^{-/-}Mdm4^{-/-}p53^{H/H}$ mouse

tissues. Based on our previous data that triple-null (*Mdm2*$^{-/-}$*Mdm4*$^{-/-}$*p53*$^{-/-}$) mice are viable, these experiments are feasible (Barboza et al. 2008).

Regulation of p53 through deubiquitination

As we mentioned earlier, just like most enzymatic reactions, ubiquitination is a reversible process. A class of enzymes has been identified that can reverse the effect of ubiquitinating enzymes and rescue p53 from degradation. These enzymes are appropriately called deubiquitinases (DUBs). Hausp (herpes-specific simplex virus associate protein), also known as USP7, the first DUB identified, is one such enzyme. HAUSP functions by deubiquitinating Mdm2 and Mdm4 (Cummins et al. 2004; Meulmeester et al. 2005). Somatic deletion of *HAUSP* in HCT116 cells leads to a dramatic increase in p53 protein levels (Cummins et al. 2004). Neural cell-specific deletion of *Hausp* results in p53-dependent neonatal lethality, suggesting a tissue-specific role of Hausp in regulation of p53 (Kon et al. 2011). However, the *Hausp* knockout mouse is embryo-lethal ~6.5–7.5 dpc due to severe reduction of cell proliferation (Kon et al. 2010). Interestingly, deletion of *p53* does not rescue this phenotype, suggesting involvement of p53-independent mechanisms.

USP10, a cytoplasmic DUB, directly deubiquitinates p53. Following genotoxic stress, USP10 is phosphorylated by ATM and translocates to the nucleus, where it deubiquitinates and stabilizes p53. Genetic ablation of *USP10* suggests it plays a central role in ROS (reactive oxygen species) regulation and enhances arsenate-induced apoptosis under both steady-state and stress conditions (Takahashi et al. 2013). Recent studies have also shown that USP10 deubiquitinates mutant p53 and thus may promote the oncogenic effects of mutant p53 in tumor cells (Yuan et al. 2010). Since USP10 deubiquitinates mutant p53, it may interfere with Mdm2 or other yet-unidentified E3 ligase-mediated ubiquitination and degradation of mutant p53. It will be interesting to test this in vivo.

Another DUB, USP42, counters the ubiquitination of p53 after genotoxic stress, thereby decreasing the response time for p53 activity (Hock et al. 2011). It does not affect the basal levels of p53 in homeostatic conditions. An animal model has not been generated.

An understanding of the physiological significance of DUBs is still in its infancy. It is not clear why an energy-exhaustive mechanism such as ubiquitination and deubiquitination would be preferred to regulate p53 activity. It could be to ensure a rapid response under conditions of need or to allow the flexibility to regulate p53 in a more tissue-specific manner. So far, in vivo evidence has not been generated to prove or disprove this.

p53 E3 ligases in evolution

Many of the ligases that we discussed above have been traced back in evolution. Mdm2 has been reported even in *Trichoplax*, a single-cell organism (Lane and Verma 2012). Biochemical analysis of Mdm2 from *Trichoplax* is awaiting confirmation that it is a bona fide p53 ubiquitin ligase. Still, the presence of an inhibitory protein highlights the importance of p53 regulation. A protein similar to Trim24 has been reported in yeast (Bodem et al. 2000). Of course, Trim24 also has an ortholog in *Drosophila*, *Bonus*, which, when targeted, results in p53-dependent apoptosis (Allton et al. 2009). Arf-Bp1 is conserved in *Caenorhabditis elegans* and *Drosophila melanogaster* (Adams et al. 2000; Ross et al. 2011). Pirh2 has a putative ortholog in *D. melanogaster* (Leng et al. 2003). Orthologs for Cop1 have not been reported in lower organisms. On the other hand, Mdm2 is not present in *C. elegans* and *D. melanogaster*, although a protein similar to p53 is present. So how do these animals regulate p53 in the absence of Mdm2? Do they express some other enzymes that can compensate for the lack of Mdm2? Species specificity of E3 ligases could also be a reason. Higher vertebrate genomes have multiple such E3 ligases. It can be speculated that higher multicellular organisms with longer life spans have a greater opportunity to be exposed to various genotoxic signals and thus need multiple E3 ligases to regulate p53. A built-in functional redundancy presents a beneficial strategy for ensuring survival of higher organisms, while simpler organisms may not require this kind of protection. Evolutionary studies will provide new insights on p53 regulation.

Why do multiple enzymes inhibit p53 functions?

The ubiquitously expressed p53 protein functions as the central node for interpreting and resolving stress responses. p53 levels are exceptionally low in most tissues under homeostatic conditions and escalate in response to stress signals. It is overly simplistic to assume that a ubiquitously expressed multifunctional protein like p53, which is so intricately involved in determining cell fate, could be regulated by a single/universal E3 ligase in multicellular organisms. Effects of p53 stabilization are also manifested in different ways depending on tissue type and developmental stage (Ringshausen et al. 2006; Zhang et al. 2014). While p53 is readily stabilized in radiosensitive tissues and some nonradiosensitive tissues (Zhang et al. 2014), in other organs such as the liver, no changes in its levels are observed. In addition, p53 stability/activity is dampened in older mice (Feng et al. 2007; Zhang et al. 2014). This implies that temporal and tissue-specific factors might be involved in regulating p53 levels. A recent study shows that *p53(ΔCTD)* mice that lack the lysine-rich C-terminal region of p53 overexpress p53 only in certain tissues and further confirms this hypothesis (Hamard et al. 2013). Possibly, a permutation and combination of multiple E3 ligases regulates p53 in various tissues in response to different types of stress signals. Post-translational modifications of p53 could also be involved in determining E3 ligase involvement. To date, only Mdm2's role in p53 ubiquitination has been established beyond doubt. Could it be that while Mdm2 functions as the critical E3 ligase, other proteins that may or may not function as standalone E3s act as either cofactors or E4s to fine-tune the p53 ubiquitination process? Mdm4 clearly falls into this category. This could

explain why most of the E3 ligases function well in in vitro systems yet fail to produce a noticeable difference in p53 stability in vivo. It can also be argued that use of saturating nonphysiological amounts of purified/transfected reaction components could skew the results in vitro. Another important point to note is that many of the E3 ligases are themselves transcriptional targets of p53. It remains possible that they are engaged in p53 regulation in response to different types of stress signals.

E3 ligases as therapeutic targets

Reintroduction of functionally active wild-type p53 negatively impacts tumor growth in mice (Martins et al. 2006; Xue et al. 2007; Wang et al. 2011; Li et al. 2014). Similarly, p53 gene therapy based on retroviral and adenoviral vectors has been assessed in non-small-cell lung carcinomas (NSCLCs) and Li-Fraumeni syndrome patients and shows some efficacy (Roth et al. 1996; Senzer et al. 2007; Shi and Zheng 2009). Enhancing the stability of p53 to prolong its activity could be another mechanism. p53 activation for therapeutic purposes has been recently reviewed in detail (Li and Lozano 2013; Wade et al. 2013). Since E3 ligases play an important role in regulating turnover of p53 and in turn its activity, they present fascinating targets for drug therapy. Treatment of culture cells with MG132, a proteasomal inhibitor, is commonly used to stabilize p53 (Maki et al. 1996). Now, the next big question is: Can this stability be used for therapeutic purposes? There is a precedent for using a proteasome inhibitor for therapeutics in human cancer with the FDA approval of proteasome inhibitor bortezomib (which acts independently of p53) for the treatment of relapsed or refractory multiple myeloma (Field-Smith et al. 2006).

A typical strategy for testing new compounds for therapeutic intervention requires them to either inhibit interaction of an E3 ligase with the p53 protein or directly alter its E3 ligase function per se. One of the best-studied such small molecules is Nutlin 3a, which binds Mdm2 and interferes with its ability to interact and degrade p53 (Vassilev et al. 2004). Nutlin 3a treatment induces apoptosis, cell cycle arrest, and senescence in culture cells and mouse xenograph models. Phase I trials have been carried out with RG7112, a nutlin-3 analog, in patients with solid tumors or leukemia (http://www.clinicaltrials.gov, NCT0062387 and NCT00559533). Other compounds, based on a similar concept such as RITA (reactivation of p53 and induction of tumor cell apoptosis) and PRIMA (p53 reactivation and induction of massive apoptosis), have also yielded encouraging results in cell culture and xenograft experiments (Bykov et al. 2002; Issaeva et al. 2004).

Multiple small molecules that can directly inhibit Mdm2 ubiquitin ligase activity have been identified. HLI98, which belongs to the family of 5-deazaflavin derivatives, is one such molecule (Yang et al. 2005). HLI98 inhibits Mdm2 and reactivates p53 in vitro and in cell-based assays. Other compounds, such as Mdm2 E3 ligase inhibitor 23 (MEL23) and MEL24 (Herman et al. 2011), inhibit Mdm2 and p53 ubiquitin conjugates in cells and increase the stability of Mdm2 and p53. Active rhodamine derivatives have also been patented for inhibition of Mdm2-mediated ubiquitination of p53. As a caution, these Mdm2 inhibitors also exhibit some degree of p53-independent cytotoxicity at higher concentrations. An in vivo test of these compounds is necessary. Nonetheless, these initial proof-of-principle studies underscore the possibility of development of more efficacious inhibitor molecules in the near future.

In addition to Mdm2, inhibitors are also being designed to inhibit Mdm4 functions. Based on mild phenotypes observed after Mdm4 inhibition in cell culture and animal models, it is argued that these may be less toxic (Garcia et al. 2011). Attempts to develop drugs that can inhibit E2 enzymes are ongoing. To achieve this, more insights about specific E2 requirements for E3 ligase function are urgently needed.

As a word of caution, prior to testing any p53-stabilizing/activating drug in clinic, the p53 mutational status in the patient needs to be evaluated. Unintended stabilization of mutant p53 can have serious side effects (Terzian et al. 2008).

Future perspectives

A series of E3 ubiquitin ligases that target p53 for degradation has been identified. For Mdm2, the data clearly show that p53 is a substrate, as loss of *Mdm2* leads to increased p53 stability/activity and cell death phenotypes. In fact, Mdm2 is such a potent inhibitor that it probably masks functions of other E3 ligases that might fine-tune p53 activities in vivo. To overcome this, in vivo models in which Mdm2 levels are low, as in hypomorphic *Mdm2* (Mendrysa et al. 2003), or in which they cannot be induced further, such as in $Mdm2^{P2/P2}$ mice (Pant et al. 2013), could be used. A combinatorial analysis of other ligases might provide insights as to their importance. For example, combinations of alleles in $Mdm2^{+/-}$ mice might unveil important roles for these ligases, as $Mdm2^{+/-}$ are compromised in some cases, such as with *Mdm4* haploinsufficiency and in response to DNA damage (Terzian et al. 2007). In vivo modeling of mutations that can specifically disrupt only the E3 ligase activity of the protein will be insightful.

On the other hand, data are less clear regarding the in vivo relevance of other E3 ligases in p53 regulation. Many of these ligases are overexpressed in cancers. Inappropriate high levels of these ligases are likely to negatively impact p53 levels and thus contribute to tumor development. Generation of conditional, tissue-specific transgenic mice could shed light on this hypothesis. Additionally, we have not exploited the use of human tumors in these analyses. Do tumors with high levels of these ligases retain wild-type p53, as do most Mdm2-overexpressing tumors?

An important question normally overlooked in the quest for p53 E3 ligases is: What about the E2 enzymes? Determining the identity of the E2s that are recruited by Mdm2 or the other E3 ligases can help in the interpretation of their biological functions. In vitro experiments have been conducted primarily with a selective subset of

E2s, UbcH5B or UbcH5C, to test the function of E3 ligases (Saville et al. 2004). What if optimum E3 ligase function requires distinct E2s in different cellular settings?

Conclusions

Tight regulation of the p53 tumor suppressor is essential for cell survival and is manifested by a complex network of ubiquitin enzymes. Some of these enzymes, Mdm2 and Mdm4 in particular, are essential, while others appear to have minor effects on p53 activities. These may still be critical regulators of p53, as small changes in p53 levels alter tumor phenotypes in vivo. Differences in p53 protein levels of as little as 7% (produced by a combination of hypomorphic and null alleles) yield significant differences in tumor onset in mice (Wang et al. 2011). Additionally, SNPs in the *Mdm2* promoter, such as SNP309 and SNP285, with small effects on Mdm2 levels alter cancer risk (Bond et al. 2004; Post et al. 2010; Knappskog et al. 2011). Thus, all ubiquitin ligases that affect p53 levels likely contribute to tumor phenotypes. The physiological and tumorigenic potential of many of these ligases has yet to be examined in detail. Particularly, robust functional assays are needed to analyze enzyme functions in close to physiological settings. As ubiquitin enzymes inhibit p53 function, analyses of genetic models in which these enzymes are overexpressed may be more informative and provide direct proof in support of their functional claims. Conditional mouse models in which the specificity of these ligases can be tested in a temporal and tissue-specific manner are also needed. The ubiquitin pathway is unequivocally crucial in regulating p53 activity, and a better understanding of the physiological contributions to cell survival and tumor development is needed. This knowledge will likely yield more therapeutic targets for patients.

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