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The Mdm network and its regulation of p53 activities: a rheostat of cancer risk

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Abstract

The potent transcriptional activity of p53 (TP53) must be kept in check for normal cell growth and survival. Tumors, which drastically deviate from these parameters, have evolved multiple mechanisms to inactivate TP53, the most prevalent of which is the emergence of TP53 missense mutations, some of which have gain-of function activities. Another important mechanism by which tumors bypass TP53 functions is via increased levels of two TP53 inhibitors, MDM2 and MDM4. Studies in humans and in mice reveal the complexity of TP53 regulation and the exquisite sensitivity of this pathway to small changes in regulation. Here we summarize the factors that impinge on TP53 activity and thus, cell death/arrest or tumor development.

Keywords

Mdm2; Mdm4; mouse models; p53; TCGA

Introduction

Cellular distress signals activate p53 (MIM#191170), and cells respond to this activation by ceasing proliferation, senescing, differentiating, or dying. Cellular proteins such as Mdm2 (MIM#164785) and Mdm4 (MIM#602704) can dampen p53 activity and allow the cell to live and propagate. The relationship between p53 and its inhibitors is dynamic and is usurped by tumor cells. This review focuses on the mechanisms that regulate p53 activity in mouse and human cancers, with particular attention to Mdm2 and Mdm4.

Inactivation of the p53 tumor suppressor pathway by multiple mechanisms

p53 is a key transcriptional regulator that controls multiple signals by activating genes with roles in cell cycle arrest, senescence, apoptosis, metabolism, differentiation, and survival

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(Vousden and Prives, 2009). Cell cycle arrest, senescence, and apoptosis are tumor suppressive mechanisms, whereas changes in metabolism often fuel tumor cell growth and survival. In addition, the ability of p53 to inhibit its own activity through Mdm2 regulates survival and allows cells no longer stressed to survive. Clearly, these activities underscore the importance of p53 in regulating cell fate decisions. Therefore, mutations that deplete TP53 activities and deletions of *TP53* (GenBank accession number NM000546) are common events in the development of most human cancers. In particular, missense mutations account for approximately 75% of all *TP53* mutations in cancer (Hamroun, et al., 2006; Olivier, et al., 2010)

The most common mechanism of altering the p53 pathway is through mutation of the *TP53* gene itself. A single base change can alter an amino acid crucial for TP53's most important biological function as a transcriptional regulator. Modification of any of five arginine residues that are hotspots for TP53 mutations in human cancers disables TP53 sequence-specific DNA binding (Cho, et al., 1994). Moreover, signature mutations in the DNA binding domain (some of which occur at these same hotspots) can arise through exposure to common insults such as ultraviolet light, smoking, and exposure to aflatoxin or other chemical carcinogens (Vogelstein and Kinzler, 1992).

TP53 missense mutations clearly predominate over frameshift and nonsense mutations in cancer cells (Olivier, et al., 2002). Thus, besides eliminating wild type TP53 transcriptional activity, another reason missense mutations are common is that these mutations lead not only to the loss of TP53 tumor suppressive functions but also to the acquisition of other activities known as gain of function (GOF) (Sigal and Rotter, 2000). Early experiments indicated that immortalized cells lacking *TP53* gained additional transforming properties upon expression of mutant TP53 proteins (Dittmer, et al., 1993). Cells expressing the most common TP53 mutants, in contrast to cells lacking *TP53*, show increased metastatic potential and invasiveness (Crook and Vousden, 1992; Hsiao, et al., 1994). In Li-Fraumeni syndrome (LFS), individuals with *TP53* missense mutations show a higher cancer incidence and an earlier age of tumor onset than individuals with truncating or splicing mutations (Birch, et al., 1998). These data are supported by studies in mice that inherit a single amino acid change in the DNA binding domain of p53 (GenBank accession number NM011640) that mimics a mutation hotspot found in human cancers (Lozano, 2007). Mutant *p53*-heterozygous mice develop many metastatic tumors that are rare in mice lacking *p53* (Lang, et al., 2004; Olive, et al., 2004). Additionally, differences in the tumor spectrum were apparent when comparing two different hotspot mutations in the 129Sv strain of mice, (Olive, et al., 2004). Thus, cells expressing mutant p53 appear to have a survival and/or growth advantage over cells lacking *p53*. These *in vitro* and *in vivo* data support the gain-of-function phenotype of mutant p53 in tumorigenesis.

Although deletion or mutation of *TP53* itself accounts for approximately 50% of the alterations in TP53, other modifications also derail the p53 pathway (Figure 1) (Vogelstein, et al., 2000). The best example is the overexpression of the TP53 inhibitors MDM2 and/or MDM4. MDM2 is highly expressed in numerous types of human tumors, and the majority of these retain wild-type *TP53* (Rayburn, et al., 2005; Wade, et al., 2013). Mdm2 is an E3 ubiquitin ligase that ubiquitylates and targets p53 for degradation (Haupt, et al., 1997;

Honda, et al., 1997; Kubbutat, et al., 1997). Under some circumstances, Mdm2 can also ubiquitylate itself and Mdm4, which leads to the opposite phenotype: p53 stability and activity (Linke, et al., 2008). The critical function of Mdm2 as a negative regulator of p53 was discovered in mouse models. Loss of *Mdm2* leads to cell death by apoptosis and embryonic lethality at the blastocyst stage, and this phenotype is completely rescued by concomitant deletion of *p53* (Chavez-Reyes, et al., 2003; Jones, et al., 1995; Montes de Oca Luna, et al., 1995).

The Mdm2-interacting protein Mdm4 (also known as MdmX) also negatively regulates the tumor suppressor function of p53 by binding and masking the amino terminal transcriptional activation domain of p53 (Shvarts, et al., 1996). Mice lacking *Mdm4* exhibit embryonic lethal phenotypes that are also completely rescued by *p53* deletion (Finch, et al., 2002; Migliorini, et al., 2002; Parant, et al., 2001). As with MDM2, MDM4 is overexpressed in many human cancers (Danovi, et al., 2004; Gembarska, et al., 2012; Han, et al., 2007; Leventaki, et al., 2012; Ramos, et al., 2001; Riemenschneider, et al., 1999; Valentin-Vega, et al., 2007; Wade, et al., 2013). Recent data indicate the *MDM4* gene is amplified in 65% of human retinoblastoma, a tumor of the eye that often results from inherited mutations in the *RBI* gene (MIM#614041) (Laurie, et al., 2006). In the same study, the recapitulation of *RBI* loss and *MDM4* overexpression in primary human retina explants yielded a retinoblastoma phenotype. Retinoblastoma was once thought to be an example of a cancer that develops without the need to inactivate TP53, because *TP53* mutations are very rare in this cancer type (Kato, et al., 1996); however, the discovery of *MDM4* amplification in the majority of retinoblastomas proved this assumption incorrect. Melanoma is another tumor in which TP53 is rarely mutated, but approximately 65% of these tumors also have high levels of MDM4 (Gembarska, et al., 2012). These data confirm an important role of MDM4 in tumorigenesis. Thus, tumors that rarely acquire TP53 mutations have developed other mechanisms to inactivate the p53 pathway.

Both Mdm2 and Mdm4 contain RING domains. However, only the RING domain of Mdm2 encodes a functional E3-ubiquitin ligase that promotes p53 degradation (Linke, et al., 2008). Mdm2 and Mdm4 also interact with each other through their respective RING domains, and this interaction is essential for inhibition of p53 (Figure 1). Genetic deletion or mutation of the Mdm4 RING domain, which disrupts interaction with Mdm2, leads to p53-dependent embryonic lethal phenotypes, which indicates the importance of the Mdm2/Mdm4 heterodimer in regulating p53 activity (Huang, et al., 2011; Pant, et al., 2011). These data indicate that Mdm4 is a key cofactor of Mdm2 and that the Mdm2-Mdm4 complex is the most efficient inhibitor of p53 activity.

Other regulators of the p53 pathway

Numerous other proteins bind Mdm2 and/or p53 and affect p53 levels and activities (Riley and Lozano, 2012). Here, we highlight the interactions that are altered in tumors that lead to inhibition of p53 activity (directly or indirectly through Mdm2) and thus, contribute to tumor development. The discovery of the alternative reading frame of the *Ink4a* locus (*ARF*; *CDKN2A*; MIM#600160) and its association with Mdm2 significantly increased our understanding of the p53 pathway and its regulation (Kamijo, et al., 1998; Kamijo, et al.,

1997; Quelle, et al., 1995; Zhang, et al., 1998). ARF is normally expressed at very low levels in cells until there is cellular distress, such as deregulation of an oncogene. Oncogenic activity transcriptionally up regulates and post-translationally stabilizes ARF, which directly binds Mdm2 and holds Mdm2 away from p53 either in the nucleolus or the nucleus (Figure 1) (Chen, et al., 2010; Eischen, et al., 1999; Llanos, et al., 2001; Weber, et al., 1999; Zhang, et al., 1998; Zindy, et al., 1998). This allows p53 activation and induction of apoptosis, cell cycle arrest, or senescence. There are conflicting reports as to whether ARF directly binds and regulates Mdm4 (Jackson, et al., 2001; Wang, et al., 2001). However, if ARF does not directly bind Mdm4, it may still regulate Mdm4 indirectly through its interaction with Mdm2 if Mdm4 is bound to Mdm2 at the time (Li, et al., 2012). Additional experiments are needed to test the influence of ARF on Mdm4, and Mdm4:Mdm2 complexes.

Studies in mice show that *Arf* does not regulate Mdm2 (and consequently p53) in proliferating tissues during development and under homeostatic conditions except germ cells (Li, et al., 2013; O'Leary, et al., 2004). However, under stressful conditions, such as when oncogenes are activated, loss of *Arf* results in deregulated Mdm2 and the inability of a cell to effectively activate p53 (Eischen, et al., 1999; Schmitt, et al., 1999; Zindy, et al., 1998). ARF is inactivated in human cancers primarily through two mechanisms. First, tumors frequently delete the entire *INK4A* locus (*CDKN2A*), which includes p14^{ARF} and p16; thus, concurrently inactivating the RB pathway, since p16 inhibits Cyclin D:CDK4/6-mediated phosphorylation of RB1 and activation of the E2Fs. The *ARF* promoter can also be methylated in malignancies, which prevents *ARF* expression, but ARF is rarely mutated in human cancers (Esteller, et al., 2000; Kim and Sharpless, 2006). *Arf* proved to be a classic tumor suppressor in knockout mouse studies. Deletion of one or two alleles of *Arf* in mice leads to tumorigenesis (primarily lymphomas and sarcomas). Tumors that develop in *Arf* heterozygous mice show loss of heterozygosity of the second allele of *Arf* (Kamijo, et al., 1999). Therefore, ARF has an essential role in preventing tumor development through regulation of Mdm2, but this function appears unnecessary in most tissues during development.

Many tumors in mice and humans inactivate either p53 or ARF, leading to the conclusion that these two proteins have an epistatic relationship (Sherr, 2006). Moreover, genetic studies have demonstrated functionally incompetent p53 in oncogene-activated tumor cells that have lost *Arf* or overexpress Mdm2. Specifically, deletion of *Arf* or overexpression of Mdm2 accelerated Myc (MIM#190080) oncogene-induced B cell lymphoma development at a rate similar to that caused by Myc overexpression and p53 deficiency. Moreover, loss of *Arf* or overexpression of Mdm2 significantly reduced the selection for p53 mutations that normally arise during Myc-induced B cell lymphomagenesis (Eischen, et al., 1999; Wang, et al., 2008).

In contrast, multiple aggressive tumors have been found to have alterations in both ARF and TP53 (Rozenblum, et al., 1997; Sanchez-Cespedes, et al., 1999; Smeds, et al., 2002). For example, approximately 40% of pancreatic ductal adenocarcinomas have mutated *TP53* and deleted *ARF* (Heinmoller, et al., 2000; Hustinx, et al., 2005; Maitra, et al., 2003). However, it is unclear whether these two alterations coexist in a single cell or that a tumor has multiple clones with separate mutations. Alternatively, gain-of-function TP53 mutants may cooperate

with alterations in ARF in the development of these tumors. Furthermore, deletion of *Arf* does not accelerate tumorigenesis in *p53*-null mice, except in a stable mixed genetic background, suggesting again that alterations of *Arf* and *p53* may not be mutually exclusive under some conditions (Eischen and Boyd, 2012; Weber, et al., 2000). For example, we evaluated *ARF* and *TP53* status in the data from The Cancer Genome Atlas (TCGA) and determined that malignancies such as squamous cell carcinoma of the lung or head and neck co-inactivate *ARF* and *TP53* in approximately 20% of these tumors (Table 1; (Cerami, et al., 2012; Gao, et al., 2013). In addition, some tumors in mice and humans contain mutant *p53* and overexpress *Mdm2*, indicating that *Mdm2* may have functions independent of *p53* (Alt, et al., 2003; Cordon-Cardo, et al., 1994; Eischen, et al., 1999; Lu, et al., 2002; Peng, et al., 2001; Sigalas, et al., 1996). Moreover, *Mdm2* transgenic mice that lacked *Arf* had an accelerated tumor development compared to mice that lacked just *Arf* (Moore, et al., 2003). *Arf* has known functions independent of *Mdm2* and *p53* (Abida and Gu, 2008; Chen, et al., 2009; Sherr, 2006; Weber, et al., 2000), so it is possible for *Arf* and/or *p53* to be inactivated and *Mdm2* to be overexpressed in the same cancer. Identification and characterization of the functions of *Arf* that are independent of *Mdm2* and *p53* are needed and will further increase our understanding of the contributions of these proteins to human cancer (*p53*-independent functions of *Mdm2* are discussed below).

Because *Mdm2* ubiquitination affects *p53* levels and activity, it is not surprising to discover deubiquitinating enzymes that also regulate the *p53* pathway. The herpesvirus-associated ubiquitin-specific protease *Hausp* (also known as *USP7*; MIM#602519) is a deubiquitinating enzyme first identified as a *p53*-interacting protein (Li, et al., 2002). However, subsequent data indicate that *Hausp* is a more important regulator of *Mdm2* function and indirectly impinges on *p53* activity. *Hausp* deubiquitinates *Mdm2*, which allows *Mdm2* to functionally keep *p53* levels low. *Hausp* loss by homologous recombination in human cell lines expressing wild-type *p53* is incompatible with proliferation (Cummins, et al., 2004). The development of a conditional loss-of-function allele of *Hausp* in mice also leads to cell death in a *p53*-dependent manner in the developing neural system of the mouse (Kon, et al., 2011). However, *p53* loss does not completely rescue the *Hausp*-null phenotype, which suggests that *Hausp* has additional functions. Thus, the physiological consequence of *Hausp* loss is destabilization of *Mdm2*, which leads to increased *p53* levels and activity (Cummins, et al., 2004; Li, et al., 2004). More recent data suggest that *Hausp* also deubiquitinates *Mdm4*, another inhibitor of *p53* activity (Meulmeester, et al., 2005). Thus, in normal cells, *Hausp* levels appear to finely tune *p53* stability and activity, whereas in tumor cells, increased *Hausp* activity may be sufficient to keep *Mdm2* and *Mdm4* stable and consequently, *p53* levels low. Our evaluation of TCGA data revealed that *USP7* is rarely amplified in human cancer (Cerami, et al., 2012; Gao, et al., 2013). However, 5.3% (40 of 760) of breast adenocarcinomas contained amplified *USP7* and of those, 75% maintained wild-type *TP53* allele status, which implies that *USP7* may have other functions related to oncogenesis. Studies examining the levels and locations of *TP53*, *MDM2*, and *Hausp/USP7* in a single tumor type are needed. In addition, the generation of *Hausp* transgenic mice would further our understanding of these relationships in tumorigenesis.

Mdm2/4 gene dosage and haploinsufficiency

Small changes in the levels of Mdm2 and Mdm4 also significantly contribute to developmental and tumor phenotypes. Although *Mdm2* or *Mdm4* heterozygosity is sufficient for homeostasis, p53 activity cannot be effectively regulated by these proteins under conditions of stress. Consequently, gamma radiation or overexpression of the Myc oncogene leads to cell death when cells are haploinsufficient for *Mdm2* (Alt, et al., 2003; O'Leary, et al., 2004; Terzian, et al., 2007). Moreover, mice carrying a hypomorphic and a null allele of *Mdm2*, which express about 30% of wild-type Mdm2 levels, have increased p53 activity and are small, lymphopenic, and radiosensitive (Mendrysa, et al., 2003). Recent results also indicate that mice lacking the p53-Mdm2 autoregulatory feedback loop are normal but succumb to hematopoietic failure upon exposure to ionizing radiation due to increased p53 activity (Pant, et al., 2013). Similar results have been observed for *Mdm4* heterozygous mice with Mdm4 haploinsufficiency resulting in increased sensitivity to gamma radiation (Terzian, et al., 2007). Notably, heterozygosity of both *Mdm2* and *Mdm4* results in lethality, with 30% of mice dying *in utero* and the rest of hematopoietic failure within 21 days after birth. The observation that these phenotypes can be rescued with deletion of a single *p53* allele indicates how precisely Mdm2 and Mdm4 regulate p53 levels (Terzian, et al., 2007). With regards to tumorigenesis, heterozygosity at the *Mdm2* or *Mdm4* locus delays tumor onset in mice carrying an Eμ-*myc* transgene (Alt, et al., 2003; Terzian, et al., 2007), and decreased *Mdm2* levels delay the appearance of intestinal tumors in *Apc^{min/+}* mice (Mendrysa, et al., 2006). *Mdm4* heterozygous mice also show a lower tumor burden in an *Rb^{+/-}* background (Fang, et al., 2013). Thus, a small difference (2-fold in these experiments) in Mdm2 or Mdm4 levels has significant *in vivo* effects on tumor phenotypes. These studies emphasize the important relationship between the Mdm proteins and p53 activity in development and tumorigenesis.

The p53 pathway is exquisitely sensitive not only to levels of Mdm2 and Mdm4, but also to the regulator of these regulators. For example, loss of just one allele of *Arf* can fully rescue the effects of *Mdm2* haploinsufficiency and reduce the rate of Myc-induced lymphoma development to wild-type levels (Eischen, et al., 2004). However, deletion of *Arf* did not rescue the radiosensitivity of Mdm2 hypomorphic mice (O'Leary, et al., 2004), suggesting the p53-inducing stimuli dictate the requirements for or the effects of Arf. In the absence of oncogene activation, when *Arf* is deleted, Mdm2 can still regulate p53 effectively. Specifically, loss of one allele of *Mdm2* significantly inhibits tumor development in mice lacking both alleles of *Arf*, which suggests that Mdm2 expression facilitates tumor development caused by the absence of Arf (Eischen and Boyd, 2012; Wang, et al., 2006). *Mdm2* heterozygosity can also significantly delay tumor development in *Arf^{-/-} p53^{+/-}* and *Arf^{+/-} p53^{+/-}* mice, but not in mice lacking both alleles of *p53* regardless of their Arf status (Eischen and Boyd, 2012). These results indicate that the effects of Mdm2 haploinsufficiency are dependent on p53. Therefore, depending on the stimulus that results in p53 activation, Arf levels may or may not be involved in the regulation of p53 (Figure 1).

More relevant to human malignancies is the finding that many tumors produce elevated levels of MDM2 and/or MDM4 (Onel and Cordon-Cardo, 2004; Wade, et al., 2013). *Mdm2* transgenic mice, in which the *Mdm2* transgene was driven from its normal promoter to

create an approximately 4-fold elevation in Mdm2 expression, have an increased incidence of cancer, but not until later in life (Jones, et al., 1998). Another study found that elevated levels of Mdm2 induce an increase in genome instability early in life in these mice, but again, cancer did not develop until well into adulthood (Lushnikova, et al., 2011). Increased levels of Mdm2 cooperated with the overexpression of Myc to accelerate lymphoma development in mice (Wang, et al., 2008). Similarly, overexpression of Mdm4 together with mutant oncogenic Ras transforms cells in culture (Danovi, et al., 2004), suggesting that Mdm4 is oncogenic. To determine whether Mdm4 is oncogenic *in vivo*, transgenic mice were generated by two different labs. Increased expression of Mdm4 in the mice in which *Mdm4* was driven from the chicken β -actin promoter led to an increase in tumor development (Xiong, et al., 2010). In contrast, Myc-tagged *Mdm4* expression from an artificial promoter in the *Rosa26* locus did not result in tumor development (De Clercq, et al., 2010). The differences in cancer incidence between the two experiments may be due to differences in Mdm4 levels. Alternatively, the Myc-tagged Mdm4 transgene may alter an Mdm4 activity essential for tumorigenesis. Thus, Mdm2 or Mdm4 overexpression in mice promotes tumor development, and reduces overall survival. Because Mdm2 and Mdm4 work together to inhibit p53, it will be necessary to accurately measure the expression of both in human malignancies and to determine how their ratios contribute to inactivation of p53 and ultimately cancer.

In humans, MDM2 and MDM4 contribute to tumor development and impact patient survival (Lenos, et al., 2012; Onel and Cordon-Cardo, 2004; Rayburn, et al., 2005). Humans with cancers that contain *MDM4* or *MDM2* amplification have lower survival rates. For example, analysis of TCGA data (Cerami, et al., 2012; Gao, et al., 2013) showed that 6.6% (24 of 363) of uterine carcinomas had amplified *MDM4*, and patients with tumors with *MDM4* amplification had lower survival rates (Figure 2A). In comparison, *Mdm2* amplification rarely occurs in uterine carcinomas (0.28%; 1 in 363). TCGA data (Cerami, et al., 2012; Gao, et al., 2013) also showed a trend toward lower survival rates in patients with glioblastoma that have amplified *MDM4* (9 of 206), but because of the low numbers of cases with *MDM4* amplification (4.4%), statistical significance was not reached. However, reduced survival rates were observed for glioblastoma patients with *MDM2* amplification, which was detected in 10.7% (22 of 206) of cases (Figure 2B). In glioblastoma, only 1 of 206 tumors amplified both *MDM2* and *MDM4* suggesting that such co-amplification is rare in this cancer. Thus, there may be a preference for overexpression of one or the other MDM family member in specific tumor types or the selection of only one (regardless of which one), and this can lead to shorter survival. Clearly, much additional research is needed to identify why a particular cancer, such as retinoblastoma (Laurie, et al., 2006), would have a bias toward *MDM4* overexpression over *MDM2* overexpression when other cancers have an equal distribution of expression of these genes.

In some human cancers, *MDM2* and *MDM4* overexpression occurs through gene amplification, but high MDM2 levels can also be achieved through increased transcription and translation (Capoulade, et al., 1998; Landers, et al., 1994). Whether *MDM4* expression is increased due to mechanisms other than gene amplification is unknown, but likely. Additional research is needed to determine when and how MDM2 and MDM4 mRNA and

protein are overexpressed during tumorigenesis. Moreover, a thorough analysis of the levels of both proteins in human cancers is needed. These studies have been hampered by technical issues with MDM2 antibodies and the numerous post-translational modifications that can occur on MDM2, which inhibit binding of some antibodies (Cheng and Chen, 2011; Eischen, 2011; Maya and Oren, 2000; Meek and Hupp, 2010; Zhang and Prives, 2001). Consequently, the frequency of elevated MDM2 protein levels in human cancers is likely significantly underestimated. Additionally, what constitutes MDM2 overexpression also needs to be re-evaluated. It is now clear that small (2- to 4-fold) increases in MDM2 protein levels, such as those that occur with *MDM2SNP309* (see below), can significantly increase tumor susceptibility (Bond, et al., 2004; Post, et al., 2010).

The gene dosage of *Mdm2* also dictates the type and the number of primary tumors that develop. Over a decade of *Mdm2* research suggests that both increased and decreased levels of *Mdm2* can alter the tumor type that emerges in various mouse models of cancer. Specifically, overexpression of *Mdm2* causes more sarcomas and fewer lymphomas to develop in *p53*-null mice (Jones, et al., 1998). *Mdm2* heterozygosity in the absence of *p53* also results in a higher frequency of sarcomas than that seen in *p53*-null mice (Eischen and Boyd, 2012; McDonnell, et al., 1999). Moreover, a third of *Mdm2*^{+/-} *Arf*^{-/-} mice develop two primary malignancies, whereas *Arf*^{-/-} mice develop a single cancer (Wang, et al., 2006). Alterations in *Mdm2* concomitant with deficiencies in both *Arf* and *p53* also reveal additional effects of *Mdm2* on tumorigenesis. Specifically, two primary malignancies develop in half of the *Mdm2*^{-/-} *Arf*^{-/-} *p53*^{-/-} mice compared to a third of the *Arf*^{-/-} *p53*^{-/-} mice, and three primary cancers are only observed in *Mdm2*^{-/-} *Arf*^{-/-} *p53*^{-/-} mice (Weber, et al., 2000). *Mdm2*^{+/-} *Arf*^{-/-} *p53*^{-/-} and *Mdm2*^{+/-} *Arf*^{+/-} *p53*^{-/-} mice have a significantly higher frequency of lymphoma and lower frequency of sarcoma compared to their *Mdm2* wild-type counterparts (Eischen and Boyd, 2012). In addition, *Mdm2*^{+/-} *Arf*^{+/-} *p53*^{+/-} mice had a lower frequency of sarcoma and a higher frequency of carcinoma than *Mdm2*^{+/+} *Arf*^{+/-} *p53*^{+/-} mice (Eischen and Boyd, 2012). Thus, *Mdm2* levels can dramatically influence the rate and type of tumors that develop. Future studies will be needed to determine the contribution of *Mdm4* to these phenotypes. These data suggest that the role of the *p53* pathway in tumor-initiating cells may differ depending on cell type, and that these differences may dictate the tumor type and the rate at which the tumor develops.

Mdm2 regulation of mutant p53

The generation of mice with *p53* missense mutations has also yielded some surprising findings regarding the stability of wild-type and mutant *p53*. Not all tumors that develop in *p53*-mutant mice had stable mutant *p53* (Lang, et al., 2004; Olive, et al., 2004). A detailed characterization of mice with one of these mutations, *p53R172H* (the amino acid numbering system is based on the initiation codon as codon 1), indicates that homozygous mutant mice had undetectable levels of mutant *p53* in normal cells, suggesting that tumor-specific alterations result in stable mutant *p53* in some, but not all, cells (Terzian, et al., 2008). Because tissue culture experiments suggested that *Mdm2* could modulate the levels of mutant *p53* as well as wild-type *p53* (Midgley and Lane, 1997), the role of *Mdm2* in mutant *p53* stability *in vivo* was examined. Loss of *Mdm2* results in mutant *p53* stability in many (but not all) cells, an increase in tumor incidence, and the development of a gain-of-function

metastatic phenotype (Terzian, et al., 2008). These results suggest that endogenous levels of Mdm2 are sufficient to keep mutant p53 levels low. The data also suggest that alterations in upstream pathways that impinge on p53 activity also contribute to the tumor phenotype. Because *p16INK4a* loss is a common event in human tumorigenesis via the Rb pathway, the role of *p16* in regulating mutant p53 stability was also examined. Loss of *p16* resulted in mutant p53 stability that was associated with increased tumor incidence and a metastatic gain-of-function phenotype (Terzian, et al., 2008). Thus, this study indicates that tumors with p53 missense mutations that are not stable resemble tumors with loss of *p53*, and the stabilization of mutant p53 is a prerequisite for its gain-of-function phenotype. Additional studies clearly show that other signals that stabilize wild-type p53, such as ionizing radiation, reactive oxygen species, and K-Ras activation, also stabilize mutant p53 *in vivo* (Suh, et al., 2011).

Modifiers of the p53 pathway

A recently discovered polymorphism in the *MDM2* gene (*SNP309*) affects the expression levels of *MDM2* (Bond, et al., 2004). The presence of a G nucleotide at *SNP309* in the *MDM2* promoter creates a better binding site for the SP1 transcription factor, and thus, results in elevated levels of *MDM2*. In Li-Fraumeni syndrome patients with germline *TP53* mutations in one allele, the presence of *MDM2 SNP309G* contributes to an earlier onset of the tumor phenotype (Bond, et al., 2004; Bougeard, et al., 2006; Ruijs, et al., 2007). The *MDM2 SNP309G* allele also associates with increased risk of breast and ovarian cancer in patients with inherited *BRCA1* and *BRCA2* mutations (Yarden, et al., 2008). In contrast, several studies, some of them with thousands of patient samples, show no association between *SNP309G* and increased tumorigenesis (Schmidt, et al., 2007; Wilkening, et al., 2007). In another study, *SNP309G* was associated with increased risk of lung cancer in never or light smokers (Liu, et al., 2008). Gender and estrogen status also cooperate with *SNP309* and may account for some of the discrepancies between data sets (Bond and Levine, 2007). An adjacent SNP (*SNP285*) was recently found to offset the impact of *SNP309* (Knappskog, et al., 2011). *SNP285C* strongly reduces binding by the SP1 transcription factor to the *MDM2* promoter, which results in lower levels of *MDM2*, and therefore, higher levels of *TP53*. *SNP285C* is associated with a reduction in the risk of breast and ovarian cancers (Knappskog, et al., 2011). Therefore, SNPs in *MDM2* can alter cancer risk in opposing ways. Since *MDM4* also inhibits *TP53* function, it will be important in the future to also evaluate SNPs in *MDM4* for their contribution to cancer.

The transgenic Mdm models discussed above accurately mimic increases in Mdm2/4 levels that affect tumorigenesis, but only partially reproduce the actual genomic changes that occur in human cancers. The identification of a high-frequency single nucleotide polymorphism (*SNP309*) in the *MDM2* promoter that increases *MDM2* levels and is associated with an increased risk of spontaneous and inherited human cancers presented an opportunity to study the role of *SNP309* in cancer (Bond, et al., 2004). Mice were, therefore, generated by introducing the human promoter sequences into the homologous murine locus to examine the direct effects of *SNP309* on tumorigenesis (Post, et al., 2010). *Mdm2^{SNP309G/G}* mice exhibit an increased risk of spontaneous tumors, and the *Mdm2^{SNP309G}* allele potentiates the tumor phenotype and alters the tumor spectrum in mice inheriting a p53R172H hotspot

mutation. These data provide causal evidence for increased cancer risk in humans with the *MDM2 SNP309G* allele. Since these mice did not contain the *SNP285C* sequence, future studies should investigate whether *SNP285C* can mitigate the effects of *SNP309G* in mice as well as humans.

The complexity and number of proteins that regulate p53 activity suggest that changes in any of these proteins may affect cancer risk. Although the inheritance of a TP53 mutation in Li-Fraumeni syndrome (LFS) patients provides a dramatic increase in cancer risk, smaller changes in TP53 levels via regulators of TP53 likely contribute to an individual's risk of developing cancer, and may explain the wide variation in tumor onset even within a TP53 mutant lineage (Balmain, et al., 2003). In fact, different combinations of null and hypomorphic *p53* alleles in mice show that as little as a 7% difference in p53 protein levels alters survival (Figure 3) (Wang, et al., 2011). Additionally, changes in p53 pathway activation could combine with other cancer-predisposing alterations (e.g., mutations in *BRCA1* or *BRCA2*) or environmental carcinogens (e.g., smoking) to increase cancer risk. In fact, smoking contributes to an increased risk of cancer in LFS individuals (Hwang, et al., 2003). Thus, combinations of polymorphisms in several genes that lead to suppression of the p53 pathway are likely to modify cancer risk.

p53 gene dosage

The most common alterations in *TP53* as opposed to other tumor suppressors are missense mutations. Approximately 80% of *TP53* mutations are missense. This observation alone suggests that TP53 mutants have additional transforming activities and mouse models with knockin alleles have provided clear evidence for this hypothesis (Lang, et al., 2004; Olive, et al., 2004). Often, a mutation of one allele of *TP53* is followed by loss of the second allele and this process eliminates wild type TP53 transcriptional activity and unmasks GOF activities of mutant TP53. However, not all tumor cells with *TP53* mutations lose their wild-type *TP53* allele. In LFS patients with *TP53* mutations, only 44% of tumors lose their wild-type *TP53* gene (Varley, et al., 1997). These data are supported by studies in mice. Approximately 50% of *p53* heterozygous mice that develop tumors under 18 months of age retain the wild-type *p53* gene (Venkatachalam, et al., 1998). The retention of a wild-type *p53* allele suggests that possibly a half dose of *p53* is insufficient to maintain normality, an unlikely possibility given that *p53* heterozygous patients and mice appear normal and only develop tumors with age, which suggests the need for additional mutations. Another possibility is that the p53 protein levels generated from one allele are sufficient under some stress conditions in many cells, but insufficient to deal with other kinds of stress signals. A third possibility is that a combination of molecular changes upstream or downstream in the p53 pathway could lead to the same end result: insufficient p53 levels or activity to effect apoptosis, senescence, or cell cycle arrest. The presence of abnormally high levels of the p53 inhibitors Mdm2 and Mdm4, for example, would preclude loss of the remaining wild-type allele. Thus, a cell with a *TP53* mutation and with increased levels of TP53 inhibitors may not need to lose the remaining wild-type *TP53* allele in the process of transformation.

The discussion above suggests that the number of modifications required to inactivate the p53 pathway may vary depending on circumstances. The nature of the p53 mutation may

also contribute to the number of changes required for transformation. Obviously, loss of *p53* predisposes a cell to tumor development. However, some TP53 mutations identified in human cancers are clearly partial loss-of-function mutations that retain the ability to activate some, but not all, TP53 targets (Resnick and Inga, 2003). For example, the rare TP53R175P mutation is able to activate the cell cycle arrest but not the apoptotic program of p53 (Ludwig, et al., 1996). Mice homozygous for the equivalent mutation develop tumors with longer latency than *p53*-null mice, which indicates that the ability to arrest the cell cycle is also a tumor-suppressive activity of p53. Further, these homozygous p53 mutant mice combined with loss of *p21*, a p53 target that encodes a cell cycle inhibitor, yields a tumor phenotype that more closely resembles that of *p53*-null mice (Barboza, et al., 2006). Thus, compounding weaker mutations may yield the same end result: inactivation of the p53 pathway. However, these combinations may be rare. Our evaluation of TCGA data in 13 different tumor types shows that *p21* (*CDKN1A*; MIM#116899) is rarely deleted or mutated; only 0.84% (18 of 2148) tumors analyzed had *p21* deletions or mutations (Cerami, et al., 2012; Gao, et al., 2013). Finally, other mutations, such as the gain-of-function mutations discussed above, would require even fewer changes. Splicing mutations may fall into either category depending on whether or not a truncated protein is made. Even a truncated p53 protein may have some gain-of-function activities.

It is important to note that different cancers alter the p53 pathway by different mechanisms. For example, 50% of lung tumors have *TP53* mutations, while 30% of osteosarcomas have *MDM2* amplification and 65% of retinoblastomas have *MDM4* amplification (Laurie, et al., 2006; Oliner, et al., 1992)(and TCGA database). So why is *MDM4* amplified in retinoblastoma while *TP53* mutations are more common in lung cancers? How do chromatin modifications affect genetic alterations? Or does the accumulation of mutations in a cell depend on what mutation occurred first? Or does the cell of origin matter? New animal models will be invaluable in addressing these questions.

p53-independent functions of Mdm2 and Mdm4

Critics have used the fact that loss of *p53* can rescue the embryonic lethality of *Mdm2* or *Mdm4* deletion (Jones, et al., 1995; Montes de Oca Luna, et al., 1995; Parant, et al., 2001) to conclude that the sole function of Mdm2 and Mdm4 is to regulate p53. However, functions in development only reveal part of the story and do not explain data generated over the past 20 years that indicate Mdm2 and Mdm4 have p53-independent functions that contribute to tumorigenesis (Melo and Eischen, 2012). For example, tumors that overexpress *MDM2* and/or *MDM4* have less TP53 activity, but some tumor cells contain both inactivated *TP53* and overexpressed *MDM2* and/or *MDM4* (Cordon-Cardo, et al., 1994; Giglio, et al., 2005; Gunther, et al., 1997; Liu, et al., 2012; Lu, et al., 2002; Peng, et al., 2001; Ramos, et al., 2001; Sigalas, et al., 1996; Suda, et al., 2011; Wade, et al., 2013; Zou, et al., 1995). Our analysis of TCGA data showed that up to 6% of specific human malignancies have amplified *MDM2* or *MDM4* (and even more have increased *MDM2* or *MDM4* mRNA expression) with concomitant *TP53* mutations (Cerami, et al., 2012; Gao, et al., 2013). These data have resulted in a line of investigation into the p53-independent functions of Mdm2 and Mdm4 that have revealed unexpected results.

Multiple independent investigations have shown that Mdm2 can contribute to tumorigenesis independent of p53. For example, deletion of *Arf* in a *p53*-null background does not alter the rate of tumor development, but deletion of *Arf* concurrently with Mdm2 overexpression accelerates tumorigenesis (Moore, et al., 2003). Mdm2 overexpression in *p53*-null mice results in an altered tumor spectrum compared to the tumor spectrum in *p53*-null mice (Jones, et al., 1998). There is increased anchorage-independent growth in cells lacking *p53* and overexpressing Mdm2 compared to cells that only lack *p53* (Dubs-Poterszman, et al., 1995). Furthermore, Mdm2 overexpression regardless of *p53* status leads to genome instability *in vivo* and *in vitro* (Bouska, et al., 2008; Lushnikova, et al., 2011). Mice that lack both *Mdm4* and *p53* have an accelerated rate of tumor development, and cells from these mice have increased genome instability (Matijasevic, et al., 2008a; Matijasevic, et al., 2008b). Together, these data indicate that there are p53-independent functions of Mdm2 and Mdm4 in cancer that need to be considered when targeting these proteins. In addition, more research is needed to fully investigate the p53-independent functions of Mdm2 and Mdm4 and their contributions to cancer development.

Small increases in Mdm2 levels in normal cells are tolerated, but higher levels are incompatible with life *in vivo* and *in vitro*, except in some cases when p53 is also inactivated (Brown, et al., 1998; Carroll, et al., 1999; De Clercq, et al., 2010; Jones, et al., 1998; Kubbutat, et al., 1997). High levels of Mdm2 result in genome instability, and this occurs even in the absence of *p53*. Specifically, there was increased polyploidy in mammary epithelial cells in *Mdm2* transgenic mice with or without *p53* (Lundgren, et al., 1997). Elevated levels of Mdm2 result in increased chromatid and chromosome breaks as well as fusions (which are indicative of chromosome breaks), and these abnormalities have also been detected in cells overexpressing Mdm2 and lacking *p53* (Bouska, et al., 2008; Lushnikova, et al., 2011). In recent years, it has been elucidated that Mdm2 directly binds to Nbs1 (MIM#602667) of the Mre11-Rad50-Nbs1 (MRN) DNA repair complex. The MRN complex was identified in an unbiased screen for novel proteins that interact with endogenous Mdm2. Binding between Mdm2 and Nbs1, but not between Mdm2 and p53, was required for Mdm2 to delay DNA break repair. This delay in DNA break repair resulted in increased genome instability and did not require the E3 ubiquitin ligase activity of Mdm2 (Alt, et al., 2005; Bouska, et al., 2008). Although the precise mechanism for the delay in DNA break repair caused by Mdm2 continues to be elucidated, multiple lines of evidence indicate that Mdm2 inhibits the early DNA damage signal that results in phosphorylation of multiple protein targets and marks broken DNA for repair (Bouska, et al., 2008; Melo and Eischen, 2012). We have also generated similar data for Mdm4 (Carrillo, et al., 2013), indicating the regulation of DNA break repair is a conserved function for the members of the Mdm family of proteins. Importantly, normal physiological levels of Mdm2 were shown to regulate Nbs1-mediated DNA break repair (Bouska, et al., 2008), which indicates that this function of Mdm2 is not aberrant and not a consequence of its overexpression in cancer. Therefore, when Mdm2 or Mdm4 is high in tumors, inhibition of both p53 and Nbs1-mediated DNA break repair would lead to increased genome instability. These important data provide new insight into another function of Mdm2 and Mdm4, whose deregulation most likely contribute to tumor development in multiple ways. In addition, this function of

Mdm2 and Mdm4 also provides a therapeutic opportunity that could be capitalized on for the treatment of human malignancies that overexpress MDM2 or MDM4.

Conclusions

The p53 pathway is central in blocking proliferation and mediating apoptosis and is thus, often altered in tumor development by various mechanisms described here. MDM2 and MDM4 are critical inhibitors of TP53 that are frequently selected for overexpression in human malignancies as a mechanism of inactivating TP53 and also likely capitalizing on TP53-independent functions. Because tumors with high levels of MDM2 and MDM4 often have wild-type TP53, it is not surprising that drug companies want to activate TP53 in these cells by disrupting the interactions of MDM proteins with TP53. Consequently, numerous drugs have been designed to activate TP53 by interfering with MDM2 and/or MDM4 binding (Li and Lozano, 2013). Although these drugs do lead to TP53 activation, cancers have a unique ability to develop resistance to drugs that cause their destruction and subsequently acquired compensatory TP53 mutations (Aziz, et al., 2011; Michaelis, et al., 2011) that make the malignancy more difficult to treat. As noted in this review, MDM2 also regulates mutant TP53 stability, and these drugs would then stabilize mutant TP53, with adverse consequences. Frequent biopsies and monitoring of patients undergoing treatments is critically important. Moreover, drugs that bind MDM2 and/or MDM4 may have other unintended consequences, such as stabilizing these proteins and causing them to be present at high levels where their TP53-independent functions may predominate and negatively impact genome stability. Future studies of these drugs will need to take these confounding factors into consideration. With the large number of questions that remain unanswered and the mechanisms that are unresolved, researchers in this field should be quite busy for years to come.

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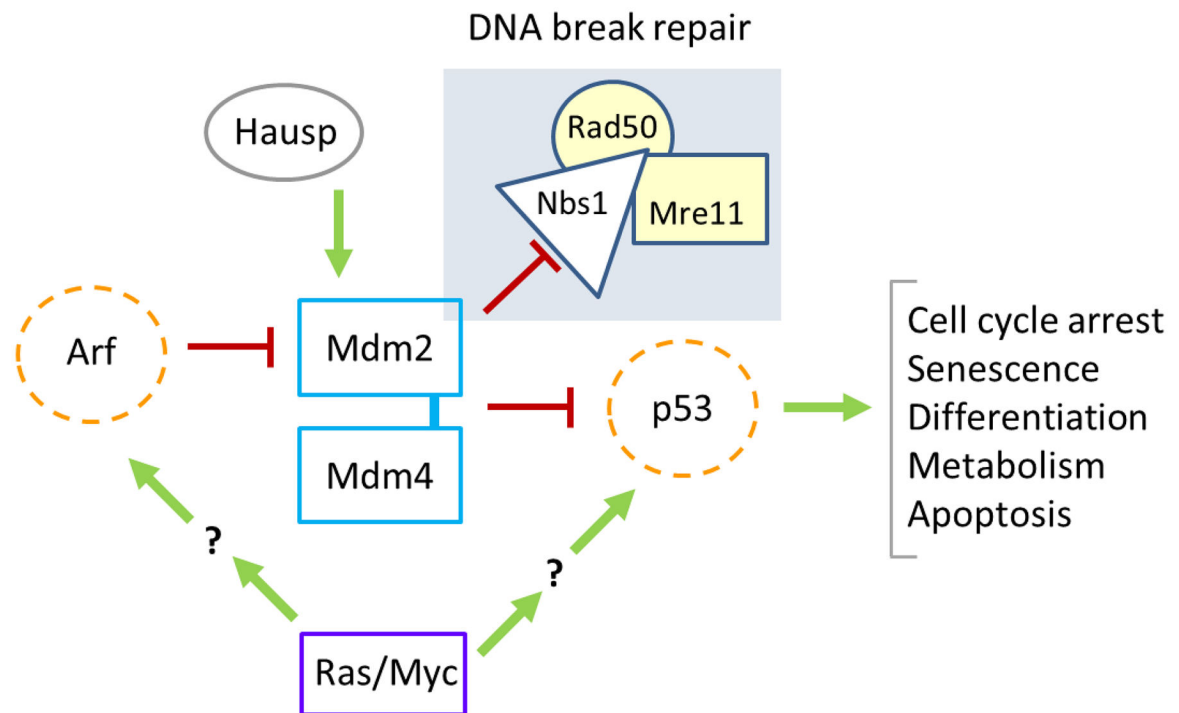


Figure 1. A model showing some of the effectors of the p53 pathway

Dotted lines denote tumor suppressors and solid lines denote activated or over produced proteins that contribute to tumor development. The shaded box denotes p53-independent functions of Mdm2. Mdm2 binds and inhibits Nbs1, a member of the MRN complex responsible for DNA break repair.

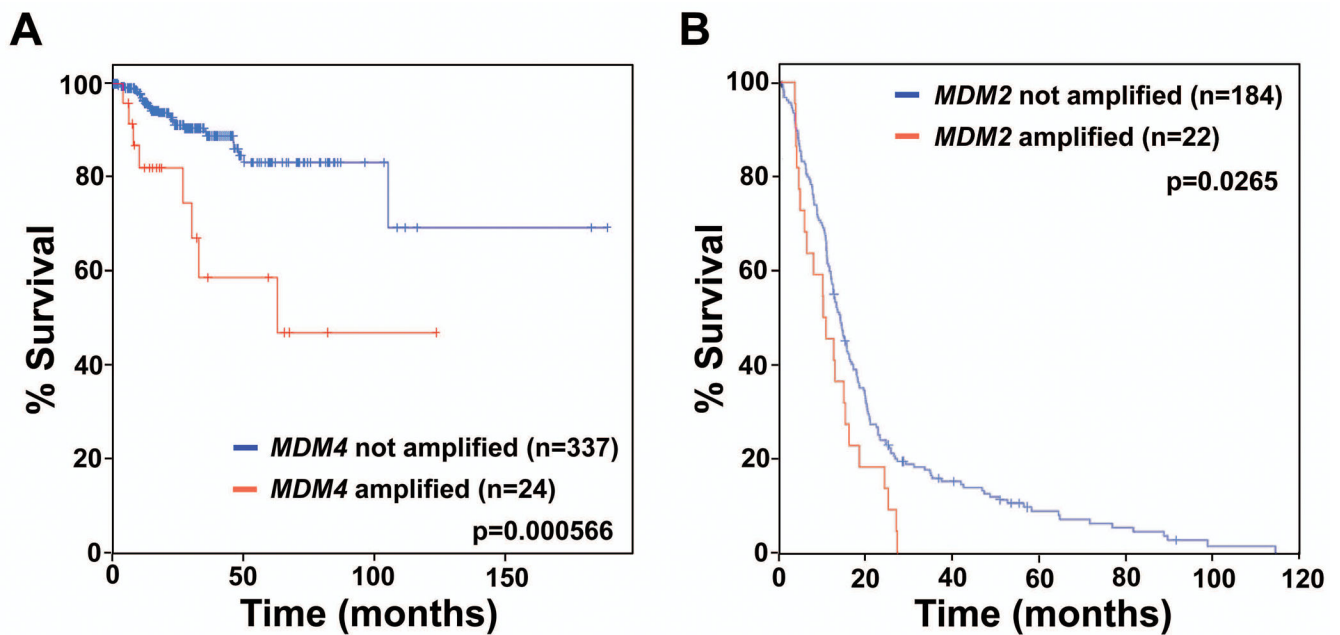


Figure 2. Amplification of *MDM4* or *MDM2* genes in specific cancers can result in decreased patient survival

Gene copy number alterations and survival data were obtained from the cBioPortal for Cancer Genomics (<http://www.cbioportal.org/public-portal/>) July 2013 (Cerami, et al., 2012; Gao, et al., 2013). Kaplan-Meier survival curves of patients with (A) uterine carcinoma with and without *MDM4* amplification and (B) glioblastoma with and without *MDM2* amplification. The number (n) of samples of each is indicated, and p values were calculated by log-rank tests comparing survival between groups.

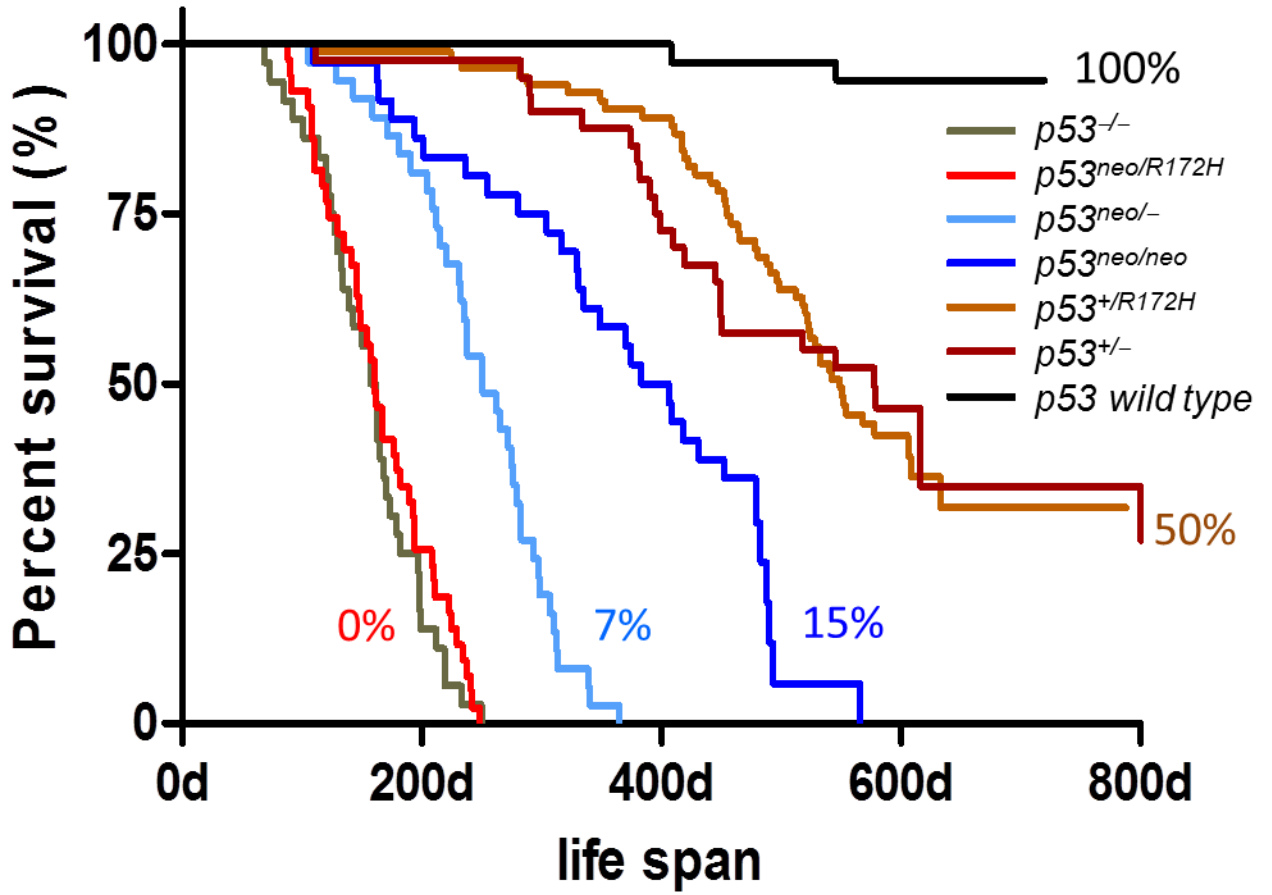


Figure 3. Small differences in p53 levels alter tumor development and survival
 Survival curves of p53 mutant mice with various alleles. The *p53neo* allele is a hypomorphic allele that expresses approximately 7% of wild type p53 levels (Wang, et al., 2011). The percentages indicated equal the levels of p53 protein detected by western blot analysis compared to the wild type, which was set at 100%. From (Wang, et al., 2011) with permission.

Table 1Frequency of co-inactivation of *TP53* and *CDKN2A* in human cancer^a

Cancer	Samples	<i>TP53</i> mut/del ^b	<i>CDKN2A</i> del ^c	Co-inactivation ^d
Acute Myeloid Leukemia	187	15 (8.0%)	2 (1.1%)	0
Adenoid Cystic Carcinoma	60	2 (3.3%)	2 (3.3%)	1 (1.7%)
Bladder Carcinoma	97	24 (24.7%)	14 (14.4%)	7 (7.2%)
Breast, Invasive Carcinoma	482	169 (35.1%)	8 (1.7%)	11 (2.3%)
Colon and Rectum Adenocarcinoma	212	108 (50.9%)	1 (0.47%)	1 (0.47%)
Glioblastoma Multiforme	236	45 (19.1%)	126 (53.4%)	27 (11.4%)
Glioma, lower grade	215	106 (49.3%)	18 (8.4%)	6 (2.8%)
Head and Neck Squamous Cell Carcinoma	302	92 (30.5%)	23 (7.6%)	60 (19.9%)
Lung Adenocarcinoma	182	76 (41.8%)	6 (3.3%)	12 (6.6%)
Lung, Squamous Cell Carcinoma	178	91 (51.1%)	5 (2.8%)	42 (23.6%)
Melanoma (Cutaneous)	225	22 (9.8%)	64 (28.4%)	3 (1.3%)
Ovarian Serous Cystadenocarcinoma	316	292 (92.4%)	1 (0.32%)	6 (1.9%)
Prostate Adenocarcinoma	103	5 (4.9%)	3 (2.9%)	0
Renal (Kidney) Clear Cell Carcinoma	418	7 (1.7%)	12 (2.9%)	1 (0.24%)
Soft Tissue Sarcoma	201	25 (12.4%)	9 (4.5%)	3 (1.5%)
Stomach Adenocarcinoma	115	43 (37.4%)	8 (7.0%)	6 (5.2%)
Thyroid Carcinoma	318	3 (0.94%)	0	0
Uterine Corpus Endometrioid Carcinoma	240	68 (28.3%)	0	0

^aData were obtained July 2013 through the cBioPortal for Cancer Genomics (Cerami, et al., 2012; Gao, et al., 2013)^bSamples that only deleted or mutated *TP53* with wild-type *CDKN2A*^cSamples that only deleted *CDKN2A* with wild-type *TP53* (mutated *CDKN2A* samples not included)^dSamples that had both a *TP53* mutation or deletion together with *CDKN2A* deletion