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REVIEW

In vivo analysis of p53 tumor suppressor function using genetically engineered mouse models

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p53 is a crucial tumor suppressor, as evidenced by the high propensity for *p53* mutation during human cancer development. Already more than a decade ago, *p53* knockout mice confirmed that p53 is critical for preventing tumorigenesis. More recently, a host of *p53* knock-in mouse strains has been generated, with the aim of either more precisely modeling *p53* mutations in human cancer or better understanding p53's regulation and downstream activities. In the first category, several mouse strains expressing mutant p53 proteins corresponding to human-tumor-derived mutants have demonstrated that mutant p53 is not equivalent to loss of p53 but additionally exhibits gain-of-function properties, promoting invasive and metastatic phenotypes. The second class of *p53* knock-in mouse models expressing engineered p53 mutants has also provided new insight into p53 function. For example, mice expressing p53 mutants lacking specific posttranslational modification sites have revealed that these modifications serve to modulate p53 responses *in vivo* in a cell-type- and stress-specific manner rather than being absolutely required for p53 stabilization and activation as suggested by *in vitro* experiments. Additionally, studies of *p53* mouse models have established that both p53-driven cell-cycle arrest and apoptosis responses contribute to tumor suppression and that activation of p53 by oncogenic stress imposes an important barrier to tumorigenesis. Finally, the use of mouse strains expressing temporally regulatable p53 has demonstrated that p53 loss is not only required for tumor development but also required for tumor maintenance, suggesting that p53 restoration in human cancer patients may be a promising therapeutic strategy. These sophisticated *p53* mouse models have taught us important lessons, and new mouse models will certainly continue to reveal interesting and perhaps surprising aspects of p53's complex biology.

Introduction

p53 is a crucial tumor suppressor gene, as evidenced by the facts that *p53* is mutated in >50% of human cancers and that deregulation of the p53 pathway occurs in tumors that retain wild-type *p53* allele (1). In addition, humans with germ line *p53* mutations are affected by the Li–Fraumeni syndrome, which is characterized by susceptibility to a broad spectrum of malignancies including breast carcinomas, bone sarcomas, brain tumors, soft tissue sarcomas and hematological neoplasms (2). Finally, an unequivocal demonstration of the importance of p53 in tumor suppression came from *p53* knockout mice, which are viable but develop tumors with short latency and 100% penetrance (3–6).

p53 suppresses tumor cell proliferation by inducing apoptosis, cell-cycle arrest or senescence in response to a variety of stresses, including DNA damage, oncogene activation and hypoxia (7,8). In unstressed cells, p53 is bound by its major negative regulator Mdm2, which promotes its rapid proteasomal degradation. Cellular stresses induce posttranslational modifications on both p53 and Mdm2, leading to disruption of the Mdm2–p53 interaction and consequent p53 stabilization and activation. In response to stress signals, p53 prevents the proliferation of damaged cells either transiently by cell-cycle arrest or permanently through apoptosis or senescence. p53 has been proposed to drive these responses by serving as a transcriptional activator to induce a host of target genes involved in cell-cycle arrest, senescence and apoptosis as well as by engaging in transcriptional activation-independent processes. Through these various mechanisms, p53 imposes an important barrier against tumor development.

Considering the importance of p53 in tumor suppression, it is not surprising that p53 has been studied extensively since its discovery in 1979. Although many experiments have utilized human cancer cell lines to study human p53, the use of mouse models has provided invaluable new insights into p53 biology, particularly because its role in tumor suppression is most appropriately studied *in vivo*. Moreover, *in vitro* cell culture conditions are very different from the environmental milieu to which cells are exposed *in vivo* and the simple process of culturing cells has been shown to trigger p53 induction, making it difficult to study p53 in physiologic conditions in culture. The use of mouse models offers unique possibilities to study p53 function both through phenotypic analysis of the whole organism and through examination of a variety of primary cell types derived from mice. The opportunity to study multiple tissues is particularly important since it has become clear in recent years that p53 function is highly cell-type and context-specific. This review will discuss the mouse models that have been generated to study p53, from classical knockout models to study *p53* loss of function to modern knock-in strategies to examine the consequences of specific *p53* mutations in a physiological context (Figure 1). These mouse strains not only have been useful for generating models of specific types of human cancers but also have provided valuable insights into p53 and cancer biology.

Modeling loss of p53 function

Three independently generated *p53* knockout mouse strains, in which sequences encoding the p53 DNA-binding domain were disrupted, demonstrated that p53 is largely dispensable for normal development, but critical for preventing tumorigenesis in different tissues (3,5,6). $p53^{-/-}$ mice invariably developed tumors, most frequently $CD4^{+}CD8^{+}$ T-cell lymphomas and sarcomas, and most succumbed to cancer by 6 months of age. These tumors were observed on a variety of different genetic backgrounds, including C57BL/6, 129/Sv, 129/Ola and BALB/c. In addition to these hallmark tumors, *p53* nullizygosity enhanced strain-specific tumor susceptibilities such as a predisposition of the 129/Sv strain to testicular tumors (9). $p53^{+/-}$ mice also developed tumors, with tumor latency intermediate between wild-type and $p53^{-/-}$ mice, and the tumor spectrum comprised more sarcomas than lymphomas. The general importance of p53 in preventing cancer was further demonstrated by crossing tumor-prone mouse strains either ectopically expressing oncogenes or deficient in other tumor suppressor genes onto a $p53^{-/-}$ background. In most of these studies, such as in *Eμ-Myc* transgenic or $Rb^{+/-}$ mice, loss of *p53* was found to collaborate in promoting tumorigenesis (9). Insights into the underlying mechanisms of the cancer-prone phenotype have come from the analysis of cells derived from $p53^{-/-}$ mice. $p53^{-/-}$ mouse embryonic fibroblasts (MEFs) were found to be immortal, failing to undergo senescence after repeated passaging *in vitro* like wild-type

Abbreviations: MEF, mouse embryonic fibroblast; PRD, proline-rich domain; Rb, retinoblastoma.

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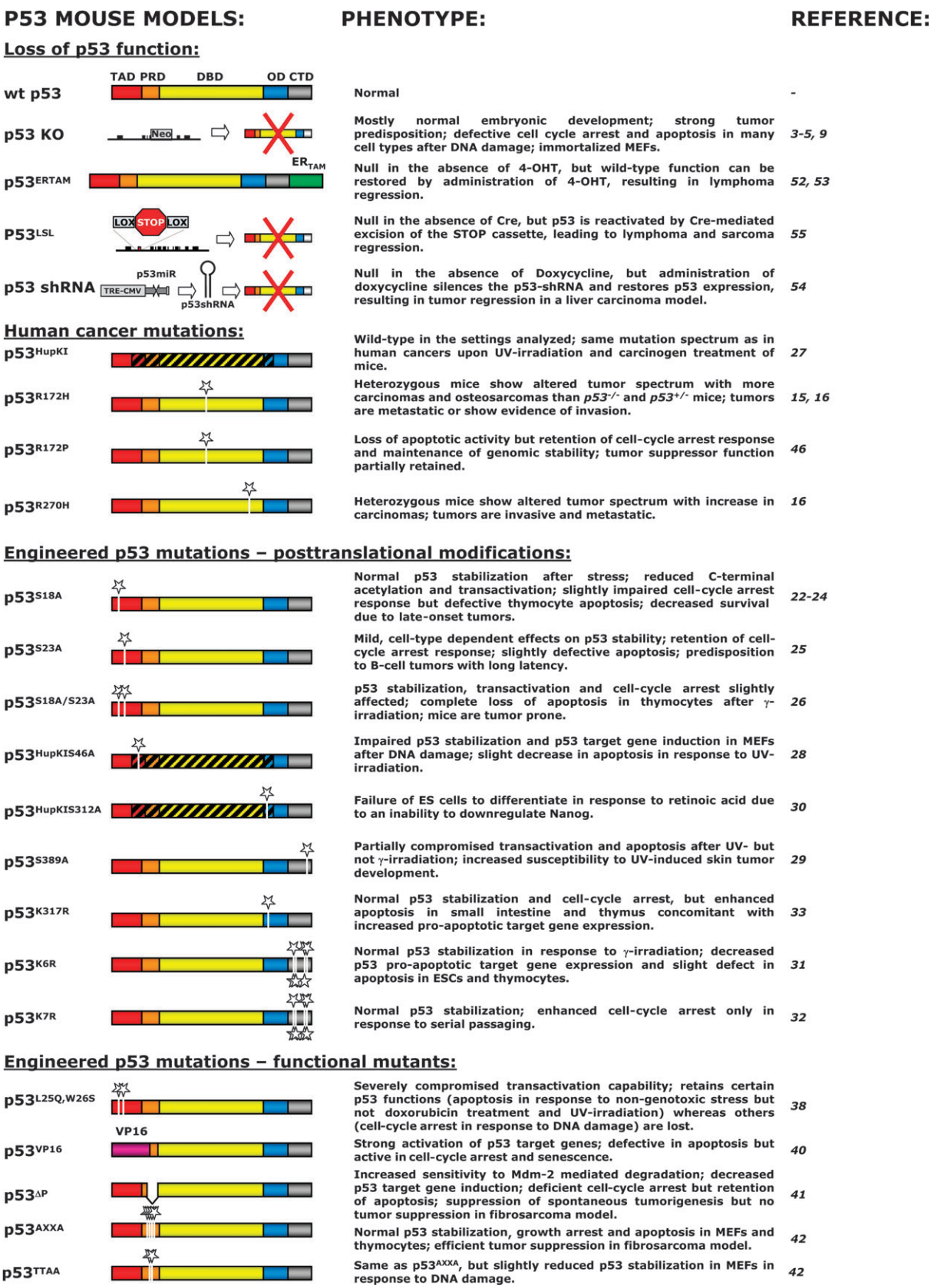

Fig. 1. Summary of the *p53* mouse models described in this review, indicating the specific modification of the *p53* locus and resulting phenotypes. Functional domains of p53: TAD = transactivation domain; PRD = proline-rich domain; DBD = DNA-binding domain; OD = oligomerization domain; CTD = C-terminal domain. Illustration of knockout, knockdown and knock-in alleles: 4-OHT = 4-hydroxytamoxifen; ERTAM = tamoxifen-regulatable estrogen receptor ligand-binding domain, HupKI = human *p53* knock-in; LSL = lox-stop-lox transcriptional stop cassette; neo = neomycin cassette; TRE-CMV = tetracycline-inducible CMV promoter; VP16 = herpes simplex viral protein 16 transactivation domain. Asterisks indicate the location of point mutations, and hatching delineates the regions of *p53* that are replaced by the human sequence in the HupKI mouse model.

MEFs (10). Moreover, $p53^{-/-}$ cells were deficient in responses to γ-irradiation: MEFs did not undergo cell-cycle arrest, and various other cell types, such as thymocytes and intestinal crypt cells, displayed compromised apoptosis (reviewed in ref. 11). These results suggest that p53 acts to prevent tumor growth by constraining cellular proliferation or inducing apoptosis in response to stress, depending on the cell type.

Although the *p53* knockout mice clearly demonstrated that p53 is an important tumor suppressor, they were not entirely satisfying as a model for human cancer because they did not exhibit the spectrum of human cancers associated with *p53* mutation. In humans, most cancers are carcinomas, whereas $p53^{-/-}$ mice developed almost exclusively T-cell lymphomas and sarcomas. In addition, some cancers that are common in Li–Fraumeni patients, such as breast cancers, were only very rarely observed in *p53* knockout mice. The influence of the genetic background on tumor susceptibility can partially account for those observed differences. For example, analysis of $p53^{+/-}$ mice on a BALB/c background revealed a high percentage of mammary gland tumors, thereby providing a model more closely resembling Li–Fraumeni patients (12). In addition, a simple explanation for the paucity of carcinomas in $p53^{-/-}$ mice is that their development is precluded because these mice succumb so rapidly to lymphomas and sarcomas. The use of conditional *p53* knockout mice, in which the *p53* coding sequences are flanked by *LoxP* sites (floxed) to allow for tissue-specific deletion of *p53* mediated by the Cre recombinase, has furthered the notion that *p53* loss can promote cancer in epithelia. For example, inactivation of *p53* in the mouse mammary gland resulted in breast cancer, suggesting that in the absence of background lymphomas, *p53* loss can facilitate the development of epithelial cancers (13). Another factor that may explain why *p53*-deficient mice do not frequently develop epithelial cancers relates to telomere biology. The mouse species commonly used in laboratories, *Mus musculus*, has chromosomes with very long telomeres, whereas telomeres of human chromosomes are much shorter. During human carcinogenesis, telomeres become critically short and cause fusion–bridge–breakage cycles, resulting in the chromosomal instability typical of human cancer cells. To determine the contribution of shortened telomeres to carcinogenesis, mice lacking the RNA subunit of telomerase were intercrossed for several generations until their telomeres were shortened sufficiently to cause genomic instability (14). *p53* deficiency in this context resulted in the development of epithelial tumors, such as breast adenocarcinomas, gastrointestinal adenocarcinomas and squamous cell carcinomas, in addition to the tumors typical of $p53^{-/-}$ mice. Therefore, differences in telomere biology and consequent effects on genome stability may contribute to the divergent tumor spectra engendered by loss of p53 function in humans and mice.

p53 mutation does not equal p53 deficiency

In contrast to *p53* knockout mice, which have lost the p53 protein altogether, the majority of human cancers carry *p53* alleles with missense mutations that allow retention of altered versions of *p53* (1). The fact that *p53* is not simply deleted or truncated in cancer cells like other tumor suppressors suggests that there may be a selective advantage for tumor cells to retain mutant p53 rather than lose p53 entirely. *In vitro* experiments have promoted the idea that mutant p53 both exerts dominant-negative effects toward wild-type p53 and exhibits gain-of-function properties (15). Moreover, early generation transgenic mouse strains carrying multiple copies of a mutant *p53* transgene driven by its own promoter expressed elevated mutant p53 in multiple tissues, and these mice developed adenocarcinomas in addition to the lymphomas and osteosarcomas observed in $p53^{-/-}$ mice, suggesting that mutant p53 exhibits gain-of-function properties, promoting the development of epithelial tumors (16). Subsequent analysis of these *p53* transgenic mice on a $p53^{-/-}$ or $p53^{+/-}$ background revealed that overexpression of mutant p53 causes accelerated tumorigenesis only in the presence of a copy of the wild-type *p53* allele, but not in $p53^{-/-}$ mice, providing the first *in vivo* evidence for a dominant-negative effect of mutant p53 (17). Although these early mouse models were highly informative, there was a possibility that overexpression of p53 might have contributed to the observed phenotypes. This issue was resolved by generating knock-in mice in which the mouse *p53* locus was replaced by mouse analogues of human *p53* cancer mutants, resulting in expression of mutant p53 from the endogenous *p53* promoter with physiologic spatiotemporal control. These mice have been useful to both study the function of mutant p53 at physiologic expression levels and more accurately model human cancers with *p53* mutations. The mutant *p53* knock-in mouse alleles included one encoding a so-called structural mutant with an altered DNA-binding domain conformation (R172H, corresponding to human R175H) and another encoding a contact mutant affecting residues that directly interact with DNA (R270H, corresponding to human R273H) (18,19). Importantly, although the survival curves of both $p53^{mut/+}$ mouse strains were similar to those of $p53^{+/-}$ mice, the tumor spectra were altered and included more frequent carcinomas, hemangiosarcomas and B-cell lymphomas (19). Remarkably, tumors arising in $p53^{mut/+}$ mice exhibited invasive properties and metastasized, unlike tumors in $p53^{+/-}$ and $p53^{-/-}$ mice. To address whether this metastatic phenotype is dependent on the wild-type *p53* allele, the two strains of *p53* mutant mice were bred to $p53^{-/-}$ mice. Again, tumor spectra of both $p53^{mut/-}$ strains were altered compared with $p53^{-/-}$ mice, and more aggressive tumors, particularly invasive and metastatic carcinomas, were observed. Taken together, these data argue against a simple dominant-negative role for p53 mutants and support the hypothesis that mutant p53 has gain-of-function properties promoting the development of carcinomas and the metastatic behavior of tumors. To investigate the underlying mechanisms for the observed differences in tumorigenesis between *p53* knockout mice and mice expressing mutant p53, the proliferation of MEFs was analyzed. $p53^{mut/-}$ and $p53^{mut/mut}$ MEFs proliferated faster than $p53^{+/-}$ or $p53^{-/-}$ MEFs, respectively (18,19), and $p53^{R172H/R172H}$ MEFs formed more colonies upon transformation by Ras than $p53^{-/-}$ MEFs (18). These studies clearly indicate that mutant p53 increases the tumorigenic potential of cells. This may be achieved through novel protein–protein interactions, such as the interaction of mutant, but not wild-type p53, with the p53-related transcription factors p63 and p73, as described *in vitro*. In contrast to p53, p63 and p73 are expressed in a more tissue-restricted manner than p53 and have specific developmental roles (20). Some studies have suggested a role for p63 and p73 in tumor suppression, and therefore, mutant p53 may exert its gain-of-function by interfering with p63 and p73 function. In support of this model, the increase in colony formation of Ras-transformed;$p53^{R172H/R172H}$ MEFs could be mimicked by knockdown of p63 and p73 in *Ras;*$p53^{-/-}$ MEFs (18). Another study proposed a distinct mechanism for mutant p53 gain-of-function, suggesting that mutant, but not wild-type p53, can interact with and inhibit proteins involved in the recognition of DNA damage, thereby interfering with the DNA damage response and promoting genomic instability (21).

Interestingly, nuclear accumulation of mutant p53 was detected in the majority of the carcinomas but not in the surrounding tissues of these mice. This finding implies that mutant p53 is stabilized specifically in cancer cells, perhaps by stresses to which cells are exposed in developing tumors or through molecular alterations that occur during tumorigenesis. These possibilities were examined in studies in which $p53^{R172H/R172H}$ mice were bred to mice lacking factors capable of destabilizing wild-type p53. $Mdm2^{-/-}$ and $Mdm2^{+/-}$ mice are deficient for the major negative regulator of p53. The tumor spectrum in $p53^{R172H/R172H}$;$Mdm2^{-/-}$ and $p53^{R172H/R172H}$;$Mdm2^{+/-}$ mice was comparable with that of $p53^{R172H/R172H}$;$Mdm2^{+/+}$ mice, but a decrease in survival and an increased frequency of metastases were noted upon *Mdm2* inactivation (22). Moreover, mutant p53 stabilization in normal tissues was enhanced when Mdm2 levels were reduced, indicating that Mdm2 can regulate not only wild-type but also mutant p53. $INK4a^{-/-}$ mice, which lack p16, a cell-cycle inhibitor acting in the retinoblastoma (Rb) pathway, were also examined, as deregulation of the Rb pathway leads to increased proliferation and induction of $p19^{ARF}$, which stabilizes p53. As with Mdm2 loss, mutant p53 was

stabilized in normal tissues, and the survival of $p53^{R172H/R172H;}$ $INK4a^{-/-}$ mice was decreased relative to $p53^{R172H/R172H}$ mice due to aggressive tumors. Together, these findings suggest that additional alterations occurring in tumor cells, such as mutations in the *Rb* pathway, can stabilize mutant p53 to promote invasion and metastasis.

Engineered mutants to define mechanisms of p53 regulation and function

Studying posttranslational modifications in vivo

Another class of knock-in mouse models has involved exchanging the wild-type allele with engineered *p53* alleles containing targeted mutations, with the goal of defining the importance of both posttranslational modifications for p53 regulation and downstream activities required for p53 responses. Not surprising for a protein that must be tightly regulated in response to a wide range of stresses and that can induce different cellular responses, p53 is subject to extensive posttranslational modifications, including phosphorylation, acetylation, ubiquitylation, sumoylation, neddylation and methylation (23). Stress- and cell-type-specific p53 posttranslational modifications influence p53 stability, cellular localization, target gene activation and, ultimately, the outcome of the p53 response. *In vitro* experiments predicted that two serines, S18 and S23 (human S15 and S20), which are phosphorylated in response to DNA damage, are particularly important for p53 activation because phosphorylation of these residues leads to disruption of the p53–Mdm2 interaction and p53 stabilization (24). Through analysis of cells derived from $p53^{S18A/S18A}$ knock-in mice, however, it was found that basal p53 levels, as well as p53 stabilization and DNA binding in response to DNA damage, were normal in $p53^{S18A/S18A}$ MEFs and thymocytes (25–27). Instead, transactivation of a subset of p53 target genes was decreased in $p53^{S18A/S18A}$ MEFs and thymocytes in response to γ-irradiation. The S18A mutation had no effect on proliferation and only slightly affected cell-cycle arrest in MEFs but caused partially defective thymocyte apoptosis after γ-irradiation. $p53^{S18A/S18A}$ mice were not as tumor-prone as $p53^{-/-}$ mice but presented decreased survival relative to wild-type mice upon aging due to late-onset lymphomas and various types of sarcomas. Thus, serine 18 plays a modest part in enhancing p53 function. Analysis of the other potential key p53 phosphorylation site, serine 23, through studies of $p53^{S23A/S23A}$ knock-in mice revealed that the effect of mutation of S23 on p53 protein stabilization by stress signals is cell-type dependent: p53 induction in response to γ-irradiation was normal in $p53^{S23A/S23A}$ MEFs but reduced in $p53^{S23A/S23A}$ thymocytes (28). In line with these observations, cell-cycle arrest in $p53^{S23A/S23A}$ MEFs was intact in response to DNA damage, but apoptosis in $p53^{S23A/S23A}$ thymocytes after γ-irradiation was impaired. In addition, the life span of $p53^{S23A/S23A}$ mice was shortened relative to wild-type mice because of a propensity to develop B-cell lymphomas. The absence of a more dramatic phenotype in these mutants with single serine alterations prompted the generation of $p53^{S18A,S23A/S18A,S23A}$ knock-in mice to test the cooperativity of phosphorylation on both S18 and S23. $p53^{S18A,S23A}$ protein stability, transcriptional activity and cell-cycle arrest activity were slightly affected in $p53^{S18A,S23A/S18A,S23A}$ MEFs (29). However, p53 stabilization was strongly decreased in $p53^{S18A,S23A/S18A,S23A}$ thymocytes in response to γ-irradiation, resulting in completely abolished apoptosis. These findings suggest that phosphorylation of both serines either is required for p53 stabilization and efficient induction of apoptosis but not cell-cycle arrest or senescence or is relevant only in specific cell types such as thymocytes. The ability of $p53^{S18A,S23A}$ expression to rescue $Xrcc4^{-/-}$ mice, which normally display lethality due to deficient DNA repair and consequent p53-dependent apoptosis, further underscores the lack of apoptotic activity of this double mutant. As expected for mice expressing an apoptosis-deficient p53 mutant, $p53^{S18A,S23A/S18A,S23A}$ mice were prone to spontaneous tumorigenesis and developed mostly B-cell lymphomas as well as leukemias, fibrosarcomas, adenomas and granulomas. Taken together, these results indicate that, rather than being universally crucial for p53 activation and function as predicted by *in vitro* experiments, these phosphorylation events may serve to modulate the p53 response in a cell-type- and context-specific manner.

The roles of several other phosphorylation sites have also been queried. Serine 46 is phosphorylated in response to DNA damage, and this event is thought to be important for the induction of apoptotic target genes and consequent cell death. Since it was unclear whether S46 was conserved in mouse p53, mice known as human *p53* knock-in (30) were used to generate $p53^{HupKIS46A/HupKIS46A}$ mutant mice (31). In $p53^{HupKI/HupKI}$ mice, exons 4–9 of mouse *p53* were replaced by the corresponding human *p53* exons, resulting in a chimeric protein containing the human p53 DNA-binding domain. p53 stabilization was impaired in UV-irradiated $p53^{HupKIS46A/HupKIS46A}$ MEFs, as were target gene induction and apoptosis. However, this mutant was not able to rescue $Xrcc4^{-/-}$ lethality, indicating that it is still capable of inducing some apoptosis. Phosphorylation of another C-terminal residue, serine 389, is specifically triggered by UV radiation and was found to increase p53 DNA-binding based on *in vitro* studies. Indeed, $p53^{S389A/S389A}$ MEFs exhibited compromised transactivation and apoptosis after UV- but not γ-irradiation, underscoring the importance of this phosphorylation site for the response to a specific stress signal (32). Although not prone to spontaneous tumorigenesis, $p53^{S389A/S389A}$ mice were more susceptible to UV-induced skin tumor development than wild-type mice. Finally, a role for a specific p53 phosphorylation event in mouse embryonic stem cell differentiation has been revealed: p53 was induced and phosphorylated on serine 315 in response to retinoic acid treatment, which is required for repression of the self-renewal factor Nanog to allow differentiation (33). p53 induction and Nanog repression were impaired in $p53^{HupKIS315A/HupKIS315A}$ embryonic stem cells, leading to maintenance of an undifferentiated state due to sustained Nanog expression. These examples further illustrate that phosphorylation of specific residues is important for p53 function in select circumstances rather than for p53 activity in general.

A well-characterized set of posttranslational modifications occurs at a group of lysine residues at the C-terminus of p53 (K367, K369, K370, K378, K379 and K383, corresponding to K370, K372, 373, K381, K382 and K386 in human p53). *In vitro* studies found that these lysines are ubiquitylated in unstressed cells and acetylated in response to stress, and they were therefore thought to be important for regulation of both p53 stability and p53 transcriptional activity. Surprisingly, however, mutation of six ($p53^{K6R}$) or seven lysines ($p53^{K7R}$, also including K384, which is not conserved in human p53) at the C-terminus of p53 merely resulted in very mild phenotypes (34,35). Basal p53 levels, p53 protein stabilization in response to stress, and tumor suppressor functions of $p53^{K6R}$ and $p53^{K7R}$ were largely comparable with wild-type p53. However, induction of certain p53 target genes was decreased in embryonic stem cells and thymocytes but not MEFs-expressing $p53^{K6R}$ in response to DNA damage. Accordingly, apoptosis of $p53^{K6R/K6R}$ thymocytes in response to γ-irradiation was slightly compromised. In the $p53^{K7R/K7R}$ cells, the only phenotypes observed were enhanced p53 stabilization at lower doses of irradiation and increased susceptibility to senesce in a 3T3 assay. One possible explanation for the lack of a severe phenotype is that individual posttranslational modifications at different lysine residues might have opposing effects, which would cancel each other in the $p53^{K6R}$ and $p53^{K7R}$ mutants lacking several lysines. For example, mutation of one lysine, K317 (corresponding to human K320), which is acetylated after DNA damage, resulted in enhanced proapoptotic gene expression and apoptosis upon γ-irradiation of radiosensitive tissues such as the thymus and small intestine, suggesting that acetylation of K317 serves to attenuate the apoptotic response of p53 (36). In addition, ubiquitylation of p53 was still detected in the $p53^{K6R}$ and $p53^{K7R}$ mutants, suggesting that other lysines in p53 can also be ubiquitylated to promote degradation. Interestingly, recent *in vitro* studies have implicated acetylation of two lysines within the DNA-binding domain, K120 and K164 (in human p53), in apoptosis and growth arrest, respectively (37). It will be important to determine the role of these residues alone or in combination with the C-terminal acetylation sites

in vivo using mouse models. Thus, contrary to expectations from *in vitro* experiments, ubiquitylation of C-terminal lysines is not a prerequisite for p53 degradation *in vivo*, and C-terminal p53 acetylation is not essential for global transcriptional activity but may serve instead to tweak p53 responses.

Elucidating functional mechanisms of p53 responses

Knock-in mice also provide a powerful approach for elucidating mechanisms of p53 action. Although transcriptional activation is an important and well-characterized p53 activity, it has been unclear whether transactivation is sufficient for inducing apoptosis, cell-cycle arrest or senescence and, ultimately, effective tumor suppression or whether additional activities of p53 are also important. p53 is a multifunctional protein that has been shown to participate in other processes such as regulating mitochondrial membrane integrity and repressing transcription (38,39). Therefore, the goal of our laboratory has been to define the molecular basis of p53 action, specifically by assessing the contribution of transactivation to p53 functions. We aimed to investigate p53 responses in a knock-in mouse strain expressing a mutant severely compromised for transactivation by virtue of introduction of two alterations into the transactivation domain (L25Q and W26S), which impair interactions with the transcriptional machinery and transactivation by p53 in cell culture assays (40). Analysis of $p53^{L25Q,W26S/L25Q,W26S}$ MEFs showed that despite nuclear localization and efficient DNA binding, $p53^{L25Q,W26S}$ was incapable of inducing robust transcription of the majority of p53 target genes, including *p21*, *Mdm2*, *Cyclin G1*, *Perp* and *Noxa* in response to DNA damage, whereas transactivation of a small subset of p53 target genes such as *Bax* was not affected (41). Consistent with the dependence of cell-cycle arrest on p21 induction, $p53^{L25Q,W26S/L25Q,W26S}$ MEFs failed to undergo arrest in response to doxorubicin treatment. For apoptosis, the situation was more complex, as $p53^{L25Q,W26S/L25Q,W26S}$ MEFs did not undergo apoptosis in response to the DNA-damaging agents doxorubicin or UV radiation but did undergo apoptosis in response to non-genotoxic stresses such as hypoxia and serum starvation. These findings suggested that mechanisms other than transactivation may be important for inducing apoptosis in these latter cases. In agreement with this notion, it was shown that in response to hypoxia, p53 failed to induce canonical p53 target genes but instead repressed gene expression (42), implying that the requirement of robust transactivation for the induction of apoptosis is stress-dependent.

In a complementary approach, we generated a *p53* knock-in strain expressing a chimeric p53 protein in which the 80 N-terminal amino acids of p53 were replaced by transactivation sequences from the Herpes Simplex Virus VP16 protein (43). The goal of these studies was to generate a p53 protein lacking transactivation-independent p53 functions that require the N-terminus of p53 while still retaining full DNA-binding and transactivation capacity. Although $p53^{VP16}$ was capable of binding to p53 response elements and potently inducing proapoptotic p53 target genes, such as *Bax* and *Noxa*, it was unable to drive apoptosis in oncogene-expressing MEFs, indicating that induction of proapoptotic target genes is not sufficient for the apoptosis response. In contrast, $p53^{VP16}$ was able to induce a strong cell-cycle arrest in MEFs, accompanied by features of senescence, further confirming that transactivation of p53 target genes is sufficient for growth arrest and senescence. Taken together, these studies suggest that although the transactivation function of p53 is sufficient for cell-cycle arrest and senescence responses, transactivation-independent functions may contribute to the apoptotic response, a notion consistent with the described roles for p53 in triggering apoptosis at the mitochondria or repressing transcription under hypoxic conditions.

The proline-rich domain (PRD) of p53 has been shown to be crucial for p53 responses *in vitro*, and therefore, a mouse strain expressing a p53 mutant lacking amino acids 75–91 comprising the PRD was generated to study the contribution of this domain to p53 function *in vivo*. Analysis of $p53^{\Delta P/\Delta P}$ cells showed that $p53^{\Delta P}$ is deficient in inducing cell-cycle arrest but is able to trigger apoptosis in E1A-expressing MEFs and thymocytes in response to DNA damage (44). Interestingly, $p53^{\Delta P}$ was able to suppress spontaneous tumorigenesis but was unable to act as a tumor suppressor in an E1A–Ras fibroblast allograft model. Thus, these findings suggest cell-type-specific differences in the mechanism of p53 tumor suppressor activity. To better define the residues within the PRD that are essential for p53 function, two additional *p53* mutant mouse strains were generated, missing either two polyproline motifs that may serve as docking sites for protein–protein interactions ($p53^{AxxA}$, with mutations P79A, P82A, P84A and P87A) or two putative binding sites for Pin1, a prolyl isomerase that regulates p53 stability ($p53^{TTAA}$, with mutations T76A and T86A). p53 accumulation in response to DNA damage was normal in $p53^{AxxA/AxxA}$ MEFs and slightly decreased in $p53^{TTAA/TTAA}$ MEFs, and both mutants exhibited normal transcriptional activity (45). Proliferation and cell-cycle arrest upon DNA damage treatment in $p53^{AxxA/AxxA}$ and $p53^{TTAA/TTAA}$ MEFs were indistinguishable from those in wild-type MEFs, as was apoptosis in $p53^{AxxA/AxxA}$ and $p53^{TTAA/TTAA}$ E1A-MEFs or thymocytes upon DNA damage. In agreement with the retained p53 responses, both p53 mutants were capable of suppressing tumor growth in an E1A–Ras allograft fibrosarcoma model. Therefore, neither protein–protein interactions through these polyproline motifs nor p53 stabilization through prolyl isomerization appear to be crucial for p53 function. It may be that critical protein–protein interactions rely on other residues within the PRD or that the PRD plays a more structural role supporting the function of the transactivation domain and DNA-binding domain.

p53 in the context of tumorigenesis

Which is the crucial cellular response elicited by p53 for suppression of tumorigenesis?

A long-standing goal in the p53 field has been to determine which cellular effector responses—apoptosis, cell-cycle arrest or senescence—contribute to p53 tumor suppression activity in different settings, and several studies in mouse models have provided insights into this question. Studies in a brain cancer model in which T_{121}, a truncated version of SV40 large T-Antigen, was expressed in the choroid plexus epithelium have been instrumental in defining the importance of apoptosis for p53-mediated tumor suppression. T_{121} binds to Rb family members, driving cell-cycle progression and tumorigenesis, which is held in check by p53. Initially, it was shown that the slow growth of T_{121};$p53^{+/+}$ tumors correlated with high levels of apoptosis, whereas the rapid growth rate of tumors in T_{121};$p53^{-/-}$ mice correlated with a deficiency of apoptosis (46). That apoptosis was in fact important for tumor suppression in this model was demonstrated by genetic analysis of mice lacking *Bax*, a p53 target gene involved in apoptosis, and by discovery that T_{121};$Bax^{-/-}$ mice displayed reduced apoptosis and a shorter tumor latency compared with controls (47). In addition, in the *Eμ-Myc* model for Burkitt's lymphoma, the kinetics of tumor development in mice lacking either p53 or components of the apoptotic pathway, such as Caspase-9, were similar, suggesting that apoptosis is key for limiting tumor growth in this setting (48). Importantly, when apoptosis was inhibited by targeting *Caspase-9*, there was no longer selection for *p53* loss, supporting the idea that inactivation of apoptosis is the means by which p53-deficiency contributes to cancer in this model. The notion that apoptosis is crucial for p53 tumor suppressor function is bolstered by the existence of human p53 cancer mutants, such as $p53^{R175P}$, that are defective in apoptosis but retain cell-cycle arrest function (29,49).

Several other mouse models indicate, however, that cell-cycle arrest and senescence are also important for tumor suppression. In particular, knock-in mice expressing the $p53^{R172P}$ mutant corresponding to the aforementioned human $p53^{R175P}$ were highly informative for proving this point. Although lacking the p53 apoptotic response, $p53^{R172P/R172P}$ mice were found to be less prone to spontaneous tumorigenesis than $p53^{-/-}$ mice, developing few thymic lymphomas as well as sarcomas only with delayed onset (49). DNA damage-induced cell-cycle arrest in $p53^{R172P/R172P}$ MEFs was largely retained,

whereas apoptosis in response to γ-irradiation in various cell types, including oncogene-expressing MEFs, thymocytes and embryonic neurons, was completely defective. In contrast to $p53^{-/-}$ MEFs, which exhibit aneuploidy, genomic stability was maintained in $p53^{R172P/R172P}$ MEFs. The importance of cell-cycle arrest was underscored by subsequent analyses of $p53^{R172P}$ on a $p21^{-/-}$ background, which resulted in defective cell-cycle arrest, chromosomal instability and accelerated tumor onset (50). Finally, $p53^{R172P/R172P}$ mice were crossed to mice deficient in the RNA subunit of telomerase to demonstrate that induction of senescence by $p53^{R172P}$ in response to telomere dysfunction is sufficient to prevent the spontaneous tumorigenesis occurring in this model in absence of p53 (51). Taken together, these studies highlight the importance of cell-cycle arrest induction and maintenance of genomic stability for p53-mediated tumor suppression.

What are the signals in developing tumors that trigger p53 activity?

Neoplastic cells experience a variety of stresses that have the potential to activate p53, including oncogene expression, DNA damage, hypoxia and nutrient deprivation. Therefore, it is important to know which signals trigger p53 responses during tumorigenesis *in vivo* to understand which pathways are involved in p53 activation. Recent studies have proposed that DNA damage is the crucial p53-activating signal since the DNA damage response pathway has been shown to be active in early neoplastic lesions, indicating that p53 might serve to prevent the expansion of cells with damaged DNA (52). DNA damage during tumorigenesis is thought to ensue from oncogene expression leading to replication fork collapse and consequent double-strand breaks, from telomere erosion, or from the action of reactive oxygen species on DNA.

Evidence also exists that oncogene expression activates p53 through $p19^{ARF}$ induction, which interferes with the p53–Mdm2 interaction and thereby stabilizes p53. In support of a central role for $p19^{ARF}$, inactivation of $p19^{ARF}$ can substitute for loss of *p53* during lymphomagenesis in the *Eμ-Myc* model for Burkitt's lymphoma (53). More recently, elegant genetic experiments taking advantage of a temporally regulatable p53 protein have bolstered the idea that $p19^{ARF}$ is critical for p53 tumor suppressor activity. $p53^{ERTAM}$ knock-in mice encode a p53 C-terminal fusion to the modified hormone-binding domain of the estrogen receptor, allowing p53 to be reversibly switched on and off by addition or withdrawal of tamoxifen. In the absence of tamoxifen, $p53^{ERTAM/ERTAM}$ mice are tumor-prone, like $p53^{-/-}$ mice (54). To assess the role of the DNA damage response in tumor suppression, p53 was restored for a short period at various timepoints after whole-body γ-irradiation. Although p53 restoration shortly after irradiation enabled a p53-dependent apoptotic response to DNA damage, it did not correlate with lymphoma suppression (55). Instead, when p53 was temporarily restored at a later timepoint, after the resolution of DNA damage, the mice surprisingly were protected from lymphomas. These findings suggest that, rather than the initial acute DNA damage, other signals present in developing tumors activate p53. Indeed, breeding of $p53^{ERTAM}$ mice onto a $p19^{ARF-/-}$ background demonstrated that protection from lymphomagenesis relied on p53 activation by $p19^{ARF}$, which is induced by oncogenic signals but not acute DNA damage. Similarly, deletion of *p53* using a floxed *p53* allele before or after γ-irradiation had little influence on tumor development and mouse survival, questioning the contribution of the acute DNA damage response and highlighting the continuous need for p53 activity for tumor suppression (56). Further supporting the idea that the response to oncogenic signaling is key for tumor suppression, mice harboring an extra copy of physiologically regulated *p53* only display enhanced protection from developing DNA damage-induced fibrosarcomas relative to wild-type mice in the presence of $p19^{ARF}$ (57). Although oncogene-induced $p19^{ARF}$ has been shown to be required for tumor suppression through these genetic studies, the importance of the p53 response to DNA damage remains to be further defined, and the role of each pathway is likely to vary according to cellular context. Moreover, the contribution of other stresses such as hypoxia or nutrient starvation to p53 activation during tumorigenesis needs further investigation.

Therapeutic potential of p53 reactivation

Is loss of p53 only required to overcome proliferative constraints and allow for the accumulation of mutations early in tumor development or do established tumors still rely on its absence? Could restoration of p53 function in tumors be a promising therapeutic strategy? These questions were addressed by several groups independently using different strategies to generate temporally regulatable *p53* alleles. In one study, analysis of $E\mu\text{-}Myc;p53^{ERTAM/ERTAM}$ mice demonstrated that sustained p53 deficiency is indeed required to maintain established tumors (58). Lymphomas were allowed to develop in the absence of tamoxifen, and subsequently, p53 function was restored by addition of tamoxifen, triggering rapid and extensive apoptosis of lymphoma cells and tumor regression. Although p53 restoration prolonged survival of the mice, they eventually succumbed to lymphomas either due to inactivation of $p53^{ERTAM}$ or due to loss of $p19^{ARF}$, which is required for p53 stabilization. The important finding that loss of p53 function is required for tumor maintenance was confirmed by two other reports. In one study, embryonic liver progenitor cells were genetically manipulated to express oncogenic $HRas^{V12}$ and a tetracycline-responsive short hairpin RNA targeting *p53*, which is expressed in the absence of doxycycline (59). Transplantation of these cells into recipient mice resulted in invasive hepatocarcinomas, but when doxycycline was added to silence the short hairpin RNA and reestablish p53 expression, the tumors completely regressed. In this case, tumor regression was mediated by senescence and, interestingly, clearance of senescent cells by the innate immune system. The third study used a *p53* allele carrying a floxed transcriptional stop element to prevent *p53* expression in the absence of Cre recombinase. Once these mice developed lymphomas and sarcomas, *p53* was restored by widespread expression of tamoxifen-inducible Cre in the mouse, resulting in the excision of the stop cassette and expression of *p53* (60). In lymphomas, the primary consequence of p53 expression was apoptosis, whereas in sarcomas, p53 expression induced senescence, highlighting the cell-type specificity of the p53 response. Consistent with the results found using the tamoxifen-regulatable p53, no detrimental effects of p53 restoration were observed in normal tissues. Therefore, established tumors depend not only on the continuous expression of oncogenes such as Hras, Kras and Myc but also on the persistent absence of the p53 tumor suppressor. Importantly, these studies also demonstrate the proof of principle that reactivation of p53 may be a promising strategy for human cancer therapy.

p53 in aging

p53 responses are certainly effective in restraining tumor development but can also have undesired consequences when p53 is activated inappropriately. This concept is illustrated by a transgenic mouse model overexpressing a truncated form of p53 called $p53^{\Delta 44}$ and a knock-in mouse strain expressing another N-terminally truncated p53 protein, which both confer increased cancer protection and accelerate aging. In both models, these phenotypes are dependent on the presence of wild-type p53, indicating that the truncated versions of p53 act to stabilize and activate full-length p53 (61,62). In contrast, mouse strains carrying a transgene comprising the entire *p53* genomic locus to ensure correct physiological regulation of expression and thus having three copies of *p53*, were more resistant to carcinogenesis without premature aging, possibly through protection from DNA damage that accumulates with age. Similar results were also obtained by increasing p53 levels either by decreasing *Mdm2* dosage or by inserting an additional copy of the $p19^{ARF}$ locus into the mouse genome (63,64). These studies indicate that increased but properly regulated p53 levels do not have the deleterious effects of premature aging. Certainly, more studies are required to define the circumstances in which inappropriate p53 responses can promote aging, a particularly important consideration for reactivation of p53 as a therapeutic strategy in cancer.

Conclusions

Clearly, mouse models have been invaluable for studying different aspects of p53 biology as well as mechanisms of tumor suppression in general. Certain predictions based on *in vitro* experiments, such as the acquisition of gain-of-function properties of mutant p53, were confirmed *in vivo*, and their contribution to tumor progression was demonstrated. In contrast, other models had to be revised, such as the role of certain posttranslational modifications for p53 function. Additionally, it has become clear that the p53 response is highly cell-type- and tissue-specific, underscoring the need for analysis of multiple cell types derived from *p53* mouse models. Although important insights have been obtained from the studies conducted this far, further exploration of both the signals that activate p53 in developing tumors and the downstream activities of p53 necessary for tumor suppression is required. In addition, although p53 restoration has been proposed as a promising therapeutic strategy, it remains to be investigated if there are undesired long-term consequences resulting from p53 activation in normal cells, and how successful this strategy would be in the presence of mutant p53. Mouse models undoubtedly will continue to be paramount for expanding and refining our knowledge of the multifaceted p53 tumor suppressor.

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