Knockin mice expressing a chimeric p53 protein reveal mechanistic differences in how p53 triggers apoptosis and senescence

Thomas M. Johnson*, Kristin Meade*[†], Navneeta Pathak*, Michelle R. Marques*[‡], and Laura D. Attardi*^{§¶}

Departments of *Radiation Oncology, [†]Pediatrics, and [§]Genetics, Stanford University School of Medicine, Stanford, CA 94305; and [‡]School of Medicine, Duke University, Durham, NC 27710

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The contribution of transcriptional activation to the p53 effector functions critical for tumor suppression, apoptosis and cellular senescence, remains unclear because of p53's ability to regulate diverse cellular processes in a transactivation-independent manner. Dissociating the importance of transactivation from other p53 functions, including regulating transcriptional repression, DNA replication, homologous recombination, centrosome duplication, and mitochondrial function, has been difficult because of overlapping motifs for these functions in the amino terminus. To determine the relative contribution of these activities and transactivation to p53 function, we generated knockin mice expressing a p53 mutant lacking domains involved in these transactivation-independent functions, while remaining competent for transactivation through fusion to the Herpes Simplex Virus VP16 transactivation domain. This chimeric mutant, termed p53^{VP16}, robustly activates the transcription of a range of p53 targets involved in both apoptosis and senescence. Intriguingly, despite being transactivation-competent, this chimeric protein shows selectivity in p53 effector function in mouse fibroblasts, with a capacity to trigger senescence but not apoptosis under a variety of conditions. Our study highlights the central role of p53 transactivation for senescence while suggesting that transactivation is insufficient for apoptosis, and provides insight into the mechanisms by which p53 serves as a tumor suppressor.

mouse model | transactivation | tumor suppression

The p53 tumor suppressor gene is a frequent target of mutation in human cancer, and loss of p53 function is frequently associated with malignant progression (1). p53's tumor suppressor activity has been attributed largely to its ability to induce apoptosis, senescence, or temporary cell cycle arrest in response to various insults, including genotoxic stresses, oncogenic signaling, and hypoxia (2). The best-characterized biochemical activity of p53 is the ability to stimulate transcription of a large cohort of target genes involved in implementing growth arrest or apoptosis (3). To function as a transactivator, p53 binds to discrete sites in the promoters and introns of genes in a sequence-specific manner and recruits numerous proteins, including components of the basal transcriptional apparatus, histone acetyltransferases, and other transcriptional cofactors essential for transcriptional initiation (4). Two discrete transcriptional activation domains in the p53 amino terminus, comprising residues 25 and 26 or 53 and 54, respectively, play key roles in interactions with these factors and in stimulating transcription (5, 6).

However, despite p53's function as a transcriptional activator being the most extensively studied facet of p53 function, the contribution of transcriptional activation to p53's role as a tumor suppressor remains surprisingly elusive. Specifically, studies using transcriptional activation-compromised p53 mutants have indicated that p53 can retain activity in the absence of transactivation potential (7, 8). Moreover, p53 has been shown to possess diverse additional activities, in regulating transcriptional

repression, DNA replication and DNA recombination, centrosome duplication, and mitochondrial membrane integrity (9–16). As with the transactivation domains, important functional motifs for these discrete activities reside in the p53 amino terminus. For example, p53 can repress transcription through interactions of residues 61–75 in the proline-rich domain with mSin3a and histone deacetylases (HDACs) (12). Additionally, p53 can regulate DNA replication and homologous recombination through interactions of residues 48–54 with Replication Protein A (13, 16) and centrosome duplication in a manner dependent on residues 13 and 17 (14). Finally, p53 can act directly at the mitochondria to induce mitochondrial membrane permeabilization through association with members of the Bcl-2 superfamily, also through the proline-rich domain (9, 10, 15). Thus, the amino terminus plays a central part in various p53 activities.

Here, to determine the relative roles of transactivation and other amino-terminal activities for p53 function, we generated knockin mice expressing a p53 mutant lacking domains involved in diverse transactivation-independent functions but still retaining p53 transactivation capacity. We removed the amino-terminal 80 aa of p53 implicated in transcriptional activation, transcriptional repression, centrosome duplication, mitochondrial function, and DNA replication/recombination, and replaced them with the transactivation domain of the Herpes Simplex Virus VP16 protein, a well characterized activation domain that can confer transactivation potential on heterologous DNA binding domains. This strategy should generate a transcriptionally active protein with the target gene specificity of p53 due to the presence of the endogenous DNA binding domain, but deficient for various other amino-terminal p53 functions. Analysis of this mutant, coupled with the knockin mouse strategy to enable analysis of primary cells with p53 expression driven by its own promoter at endogenous levels, provides an effective approach for clearly defining the requirements for p53 function in its effector pathways important for tumor suppression, apoptosis and senescence. Intriguingly, we find here that this mutant uncouples the p53 senescence and apoptotic responses, thereby providing new insight into the mode of p53 action in tumor suppression.

Results and Discussion

By replacing the amino-terminal ≈80 aa of p53 with the VP16 transactivation domain, we generated a transcriptionally active protein with the target gene specificity of p53 due to the

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[¶]To whom correspondence should be addressed. E-mail: attardi@stanford.edu.

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Fig. 1. Generation of $p53^{VP16}$ mice. (A) Diagram illustrating the homologous recombination strategy used to generate the $LSL-p53^{VP16}$ allele. A targeting vector comprising a Lox-stop-Lox transcriptional stop cassette upstream of a fusion of the VP16 activation domain to exon 4 of p53 and an HSV-thymidine kinase negative selection cassette was introduced into ES cells. PCR primers used for genotyping are noted by lowercase letters a–c. Restriction sites, expected fragment sizes, and the probe used in the Southern blotting described in B are indicated. Diagram is not to scale. (B) (Left) Southern blotting on EcoRI-digested genomic DNA identifies ES cells correctly targeted at the 5' end of the locus. (Right) Representative PCR from $LSL-p53^{VP16}$ mouse genomic DNA showing the presence of the transcriptional stop element in the $p53$ locus and verifying germ-line transmission. The upper band corresponds to the product generated from primers a and b in the wild-type allele (see A), and the lower band corresponds to the product generated from primers a and c. L, DNA ladder with indicated sizes of bands. (C) Representative example of p53 immunostaining of homozygous $LSL-p53^{VP16}$ MEFs. Cells infected with Ad-Cre or Ad-empty for 24 h were fixed and stained for p53 protein expression. (D) Western blot for p53 in homozygous $LSL-p53^{VP16}$ and $LSL-p53^{wt}$ MEFs. Cells were infected with Ad-Cre or Ad-empty for 24 h, and then either treated with 0.2 μ g/ml doxorubicin for 8 h or left untreated. Gapdh serves as a loading control.

retention of the p53 DNA binding domain, but bereft of other amino-terminal functional motifs. We generated a targeting vector carrying this $p53^{VP16}$ mutant and a transcriptional stop cassette flanked by *LoxP* recombination sites (lox-stop-lox, LSL) upstream of the $p53$ coding region to silence the allele until Cre recombinase introduction (Fig. 1A). Because this chimera cannot bind the E3 ubiquitin ligase Mdm2, which normally interacts with p53's amino terminus and plays a critical role in p53 down-regulation, inclusion of the stop element should prevent embryonic lethality associated with unrestrained p53 in the developing embryo (17, 18). The targeting vector was introduced into ES cells, and correctly targeted clones were used to generate mice (Fig. 1B).

To analyze this mutant, we used mouse embryo fibroblasts (MEFs), a readily manipulable cell type in which p53 effector functions are well characterized. After generation of homozygous $LSL-p53^{VP16}$ and control $LSL-p53^{wt}$ (19), we infected cells with either adenoviruses expressing Cre (Ad-Cre) to excise the stop element, or as a control, empty adenoviruses, leaving the cells effectively p53 null. Greater than 95% of cells expressed detectable p53 protein, as determined by immunostaining of Ad-Cre-infected MEFs (Fig. 1C; data not shown). To compare

Fig. 2. $p53^{VP16}$ binds p53 response elements and potentially transactivates a range of p53 target genes. (A) Electromobility shift assay with use of oligonucleotides corresponding to the p53 responsive element from the $p21$ promoter. Cell extracts were generated from homozygous $LSL-p53^{VP16}$ and $LSL-p53^{wt}$ MEFs infected with Ad-empty or Ad-Cre and either treated with 0.2 μ g/ml doxorubicin (dox) or left untreated. Specific competitor (sc): 20- and 100-fold excess of unlabeled oligonucleotides representing the wild-type p53 responsive element in the $p21$ promoter. Mutated competitor (mc): 100-fold excess of unlabeled oligonucleotides mutated in the critical "CwvWG" sequences of the p53 responsive element. (B) Northern blot showing the effects of $p53^{VP16}$ expression on multiple p53 target genes. RNA was prepared from cells treated as in A. For this experiment, RNA was also isolated from wild-type MEFs ($p53^{+/+}$). The same blot was stripped and reprobed for each target. Gapdh serves as a loading control.

levels of wild-type p53 and $p53^{wt}$ proteins, we performed Western blotting. Endogenous p53 is found at low levels in the cell because of proteasomal degradation mediated by Mdm2, and is stabilized in response to cellular stresses such as DNA damage (20, 21). Accordingly, in homozygous $LSL-p53^{wt}$ MEFs, p53 protein was absent in Ad-empty-infected cells and nearly undetectable on Ad-Cre infection, but highly up-regulated in cells infected with Ad-Cre and treated with the DNA-damaging agent doxorubicin (Fig. 1D). As expected for a p53 mutant unable to bind Mdm2, the $p53^{VP16}$ mutant was stabilized in Ad-Cre-infected MEFs even in the absence of DNA damage, accumulating to levels similar to those of wild-type p53 after DNA damage (Fig. 1D). These studies establish this system as an effective one in which to compare the function of the $p53^{VP16}$ protein with wild-type p53.

To examine the potential of the $p53^{VP16}$ chimera to activate p53 target gene expression, we evaluated its ability to both bind DNA and induce a panel of canonical p53 target genes. In electromobility shift assays, $p53^{VP16}$ retained the ability to bind in a sequence-specific manner to p53 response elements from the $p21$ and $Perp$ regulatory regions (Fig. 2A; data not shown). To evaluate the transactivation capacity of this mutant, we examined endogenous target gene expression by Northern blot analysis. Whereas MEFs expressing wild-type p53 displayed modest induction of genes such as $p21$, $Noxa$, and $Perp$ in a basal state and significant up-regulation in response to DNA damage, MEFs expressing $p53^{VP16}$ exhibited strong induction of a broad range of p53-regulated genes, including genes involved in cell cycle arrest, negative feedback regulation, and apoptosis, even in the absence of DNA damage (Fig. 2B). Thus, this mutant can bind to various p53-responsive elements and efficiently transactivate endogenous target genes, validating its competency as a transactivator. Furthermore, these data indicate that the specificity of p53 activation of its target genes relies on the DNA binding domain rather than the transactivation domain.

Fig. 3. p53^{VP16} is unable to induce cell death in a variety of contexts. (A) Homozygous *LSL-p53*^{VP16}, *LSL-p53*^{wt}, or *p53*^{+/+} MEFs were infected with the indicated adenoviruses for 24 h, and apoptosis was measured at the noted times after infection by Annexin/PI staining and flow cytometry analysis. The percentages of viable cells are graphed over time, and the data are derived from three independent experiments. (B) Homozygous *LSL-p53*^{VP16}, *LSL-p53*^{wt}, or *p53*^{+/+} MEFs were infected with Ad-Cre or Ad-empty for 24 h, treated with 20 J/m² UVC radiation, and examined for apoptosis at 24 h by Annexin/PI staining. The data are derived from four independent experiments. (C) Myc-expressing homozygous *LSL-p53*^{VP16} or *LSL-p53*^{wt} MEFs were infected with Ad-Cre or Ad-empty for 24 h and were then left untreated or treated with 0.5 μ g/ml doxorubicin for 24 h, followed by Annexin/PI cell death assays. For A–C, error bars represent standard deviation, and the effective *p53* genotypes examined are indicated in red.

To assess the biological capabilities of the p53^{VP16} mutant, we initially examined its ability to induce apoptosis, an activity of p53 essential for its tumor suppressor function *in vivo* (22, 23). Although untransformed MEFs are typically refractory to apoptosis, we hypothesized that because the p53^{VP16} mutant can potentially transactivate a variety of proapoptotic p53 target genes in the absence of a cellular stress, it may elicit an apoptotic response without a sensitizing stress signal. However, we found that p53^{VP16}-expressing cells displayed minimal cell death, suggesting that robust transactivation of proapoptotic p53 target genes, such as *Bax*, *Perp*, and *Noxa*, is insufficient to induce MEFs to undergo apoptosis (Fig. 3A).

The inability of p53^{VP16} to display apoptotic activity may reflect a requirement for a collateral signal to sensitize MEFs to undergo apoptosis. Specifically, MEFs either treated with UV light or expressing oncoproteins, such as c-Myc or adenoviral E1A, and exposed to various stresses are highly sensitized to undergo p53-dependent apoptosis (24–26). We found that, although MEFs expressing wild-type p53 exhibited significant apoptosis in response to UVC, MEFs expressing p53^{VP16} displayed levels of cell death equivalent to those lacking p53 (Fig. 3B). Thus, even in the context of DNA damage signaling, the p53^{VP16} mutant is incapable of inducing apoptosis. We next evaluated the ability of p53^{VP16} to induce apoptosis in c-Myc-expressing MEFs treated with doxorubicin. Myc-MEFs expressing wild-type p53 underwent efficient cell death, whereas those expressing the p53^{VP16} mutant were unable to undergo apoptosis (Fig. 3C). Thus, despite being capable of potentially up-regulating proapoptotic p53 target genes, the p53^{VP16} mutant is unable to induce apoptosis in a variety of contexts, suggesting that transactivation capacity is not sufficient for p53-mediated apoptosis

Fig. 4. p53^{VP16} induces cellular senescence in the absence of cellular stress. (A) BrdU-labeling indices were determined from homozygous *LSL-p53*^{VP16}, *LSL-p53*^{wt}, or *p53*^{+/+} MEFs infected with the indicated adenoviruses, treated with 0.2 μ g/ml doxorubicin (dox) or left untreated, pulsed for 4 h with BrdU, and analyzed by immunostaining for BrdU. Error bars represent standard deviation between three independent experiments. (B) Representative growth curve of *LSL-p53*^{VP16} or *LSL-p53*^{wt} MEFs infected with Ad-Cre or Ad-empty. Cells were cultured for the indicated length of time. This graph is representative of five independent experiments on two independently derived lines of *LSL-p53*^{VP16} MEFs. (C) Representative photomicrographs of SA- β gal staining of *LSL-p53*^{VP16} or *LSL-p53*^{wt} MEFs infected with Ad-Cre or Ad-empty. Cells were cultured for 4 days postinfection, fixed, and stained for SA- β gal activity. As a positive control, *LSL-p53*^{wt} MEFs infected with Ad-Cre and treated with 0.2 μ g/ml doxorubicin were cultured for 10 days and then stained for SA- β gal. Photomicrographs are representative of five independent experiments. (D) Western blot for PML expression. *LSL-p53*^{VP16} MEFs were infected with Ad-empty or Ad-Cre for 24 h, and then harvested 24 or 72 h later. Gapdh serves as a loading control.

in MEFs, and that other functions may be critical for p53 to trigger cell death.

Another critical means by which p53 limits the expansion of neoplastic cells is through induction of a permanent cell cycle arrest or cellular senescence (27–29). We examined the ability of p53^{VP16} to elicit a growth arrest in response to doxorubicin by using BrdU incorporation assays to assess S-phase entry. MEFs expressing wild-type p53, but not treated with DNA damage, showed an $\approx$ 2-fold decrease in the BrdU-labeling index relative to cells lacking p53 (Fig. 4A). On DNA damage treatment, cells expressing wild-type p53 halted cell cycle progression, as noted by the virtual absence of BrdU-positive cells. Interestingly, p53^{VP16}-expressing MEFs exhibited a dramatic cell cycle arrest even in the absence of DNA damage. Thus, this transactivation mutant, although unable to induce apoptosis, can enforce a potent cell cycle arrest. That this arrest is specific and relates to sequence-specific DNA binding was verified by using a DNA binding domain mutant of p53 (R248W), which acts as a dominant negative to inhibit wild-type p53 binding to DNA (30). When the p53^{R248W} mutant was introduced into p53^{VP16}-expressing MEFs, the ability of p53^{VP16} to arrest cells was abrogated, indicating that p53^{VP16} triggers arrest through DNA binding (Fig. 5).

We demonstrated that this arrest reflects a senescence response, because p53^{VP16}-expressing MEFs displayed a prolonged

Fig. 5. Expression of a DNA binding mutant, dominant negative p53 protein abolishes the cell cycle arrest elicited by p53^{VP16}. Homozygous *LSL-p53^{VP16}* MEFs were infected with retroviruses expressing the p53^{R248W} mutant (dominant negative or DNp53), and 4 days later were infected with adeno-Cre or Adeno-empty for 24 h. Cells were then pulsed with BrdU for 4 h, fixed, and stained for BrdU incorporation and p53 expression. As seen in the graph on the left, cells expressing both p53^{R248W} and p53^{VP16} incorporated high levels of BrdU, similar to the levels seen in cells expressing no p53 protein (*LSL-VP16* + empty) or only p53^{R248W} (*LSL-VP16* + empty + DNp53), suggesting that no cell cycle arrest occurred. In contrast, cells expressing only p53^{VP16} (*LSL-VP16* + Cre) showed very minimal BrdU incorporation, indicative of a strong cell cycle arrest. Representative images of the data graphed on the left are shown on the right. The bottom two rows of images use an antibody raised against the VP16 transactivation domain to show that the p53^{VP16} protein is expressed in cells also expressing the p53^{R248W} mutant.

growth arrest and exhibited robust staining for senescence-associated β -galactosidase (SA- β gal; Fig. 4B and C). Notably, this response was rapid, manifested within 4 days, and occurred in the absence of DNA damage or oncogenic signals. Additionally, p53^{VP16}-expressing MEFs displayed augmented promyelocytic leukemia (Pml) levels, another hallmark of senescence (31), compared with p53-deficient MEFs (Fig. 4D). Thus, in the absence of exogenous stress, the p53^{VP16} mutant is competent to elicit a potent cell cycle arrest recapitulating various features of senescence. These findings highlight the importance of transactivation of target genes, rather than other amino-terminal domain activities, for the p53 senescence program.

Because the p53^{VP16} mutant induced such a vigorous growth arrest in untransformed MEFs, we evaluated its ability to trigger senescence in MEFs expressing oncogenes to determine whether a p53-mediated growth arrest is capable of preventing the expansion of cells with tumorigenic potential. We first found that Myc-MEFs expressing the p53^{VP16} mutant did not proliferate, and once again stained positive for SA- β gal, in contrast to Myc-MEFs expressing wild-type p53, indicating that even in the context of a strong oncogenic signal, potent transactivation by p53 engenders a senescence response (Fig. 6A). We then examined the activity of p53^{VP16} in the context of another oncoprotein, adenoviral E1A, which disrupts the G₁ cell cycle checkpoint by inhibiting the Rb family of proteins (32). Interestingly, on measuring S-phase entry, we found that E1A-MEFs expressing p53^{VP16} exhibited BrdU positivity to levels commensurate with those seen in cells expressing wild-type p53, and failed to stain positive for SA- β gal after 4–8 days in culture (Fig. 6B and C; data not shown). Thus, the ability of this p53^{VP16} chimera to trigger cellular senescence strictly depends on the integrity of Rb family proteins to impose a cell cycle checkpoint.

Fig. 6. p53^{VP16} can induce senescence in the presence of an oncogene that stimulates the activity of proliferation enhancing pathways, but not an oncogene that sequesters cell cycle checkpoint proteins. (A) SA- β gal staining of Myc-expressing *LSL-p53^{VP16}* or *LSL-p53^{wt}* MEFs infected with Ad-Cre or Ad-empty and cultured for 4 days. (B) BrdU immunostaining on E1A- and CreER-expressing *LSL-p53^{VP16}* or *LSL-p53^{wt}* MEFs treated with 4-hydroxytamoxifen (4-OHT) or vehicle for 24 h, followed by a 4-h pulse with BrdU. Error bars denote standard deviation between three independent experiments. (C) SA- β gal staining of MEFs treated as in B, but cultured for 6 days posttreatment with 4-OHT or mock treatment. (D) Annexin/PI flow cytometry assay on E1A- and CreER-expressing *LSL-p53^{VP16}* or *LSL-p53^{wt}* MEFs treated with 4-OHT or vehicle for 24 h, followed by treatment with 0.2 μ g/ml doxorubicin (dox) for 24 h, or mock treatment. Error bars represent the standard deviation between four independent experiments. (E) p53^{VP16} retains transactivation capacity in E1A-MEFs. RNA was prepared from E1A-expressing *LSL-p53^{wt}* or *LSL-p53^{VP16}* MEFs treated with 4-OHT for 24 h or left untreated, followed by 8 h of treatment with doxorubicin or mock treatment. Northern blotting of RNA samples shows that p53^{VP16} retains robust transactivation potential on the p53 targets *p21* and *Bax*. *Gapdh* serves as a loading control.

It remained possible that the apoptotic defect we detected in cells expressing p53^{VP16} was not due to inherent deficiencies in this protein's ability to induce cell death, but instead, was the result of the rapid growth arrest induced by p53^{VP16} inappropriately driving the cell fate decision away from the apoptotic program. We therefore evaluated the ability of p53^{VP16} to induce apoptosis in the context of E1A expression, a setting in which p53^{VP16} cannot elicit growth arrest. On doxorubicin treatment, we found that E1A-p53^{VP16}-MEFs still displayed a marked apoptotic defect, with levels of cell death significantly lower than those seen in E1A-MEFs expressing wild-type p53 (Fig. 6D). Importantly, the p53^{VP16} protein retained its ability to transactivate p53 target genes in these E1A-expressing MEFs (Fig. 6E). Thus, even in fibroblasts competent to proliferate, p53^{VP16} cannot induce apoptosis.

To examine the activity of p53^{VP16} in an *in vivo* setting, we assessed the consequences of p53^{VP16} expression during development. *Mdm2*^{−/−} mice have been shown to display early embryonic lethality because of the failure to down-regulate p53 (18). Given that p53^{VP16} cannot bind Mdm2 and can induce senescence in some settings, its expression during development may induce embryonic lethality similar to that seen in mice

against p53 (Ab-1 and Ab-3, Oncogene Science) and Gapdh (Research Diagnostics) at 1:500 and 1:10,000 dilutions, respectively. For immunofluorescence we used p53 antibodies (CM5, Novocastra) at a 1:150 dilution and VP16 transactivation domain antibodies at 1:750 (BD Biosciences). Pml antibodies were the generous gift of S. Lowe and E. DeStanchina (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and used at 1:1000.

Apoptosis, Proliferation, and Senescence Assays. Annexin/PI cell death assays were performed as described in ref. 19, with the following modification. For the assays involving apoptosis in E1A-MEFs, 4-OHT was added to the culture medium (where indicated) for 24 h, then this medium was removed and replaced with medium containing doxorubicin (or vehicle) for 24 h, and apoptosis was analyzed. BrdU immunostaining was performed as described in

ref. 19. Growth curves were performed by using a crystal violet staining protocol, as described in ref. 29. SA- β gal staining was performed as described in ref. 39.

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