Protein phosphatase 5 and the tumor suppressor p53 down-regulate each other’s activities in mice

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Protein phosphatase 5 (PP5), a serine/threonine phosphatase, has a wide range of biological functions and exhibits elevated expression in tumor cells. We previously reported that pp5-deficient mice have altered ataxia-telangiectasia mutated (ATM)-mediated signaling and function. However, this regulation was likely indirect, as ATM is not a known PP5 substrate. In the current study, we found that pp5-deficient mice are hypersensitive to genotoxic stress. This hypersensitivity was associated with the marked up-regulation of the tumor suppressor tumor protein p53 and its downstream targets cyclin-dependent kinase inhibitor 1A (p21), MDM2 proto-oncogene (MDM2), and phosphatase and tensin homolog (PTEN) in pp5-deficient tissues and cells. These observations suggested that PP5 plays a role in regulating p53 stability and function. Experiments conducted with p53\textsuperscript{+/+}, pp5\textsuperscript{++/+} or p53\textsuperscript{-/-}pp5\textsuperscript{-/-} mice revealed that complete loss of PP5 reduces tumorigenesis in the p53\textsuperscript{+/+} mice. Biochemical analyses further revealed that PP5 directly interacts with and dephosphorylates p53 at multiple serine/threonine residues, resulting in inhibition of p53-mediated transcriptional activity. Interestingly, PP5 expression was significantly up-regulated in p53-deficient cells, and further analysis of pp5 promoter activity revealed that p53 strongly represses PP5 transcription. Our results suggest a reciprocal regulatory interplay between PP5 and p53, providing an important feedback mechanism for the cellular response to genotoxic stress.

Maintenance of genomic stability is critical for cell growth and survival. Many genetic disorders, including most human cancers, are associated with some form of genomic instability. The tumor suppressor gene p53 is known to play a critical role in the maintenance of genomic stability in response to various cellular and genotoxic stress factors, including DNA cross-linking agents (1), oxidative stress (2), UV/ionizing irradiation (3), and persistent DNA damage (4). p53 is a transcription factor that can repress or induce many genes in response to genotoxic stress. Mdm2 is involved in an autoregulatory feedback loop that down-regulates p53 upon the conclusion of DNA repair for reentry into the cell cycle. Mdm2 functions in a ubiquitin ligase complex that is important for nuclear export of p53 and eventual destabilization of p53.

Upon encountering genotoxic stress, the ataxia-telangiectasia mutated (ATM)\textsuperscript{2}/ATM and Rad3-related (ATR) kinases and CHK1/2 kinases are activated and subsequently phosphorylate p53. These phosphorylation events have been shown to prevent the interaction between p53 and MDM2 and subsequently lead to an increase in p53 protein level and activity (5, 6). In contrast to the well studied mechanism of p53 phosphorylation, dephosphorylation of p53 remains poorly understood. Several protein phosphatases, including protein phosphatase 2A (PP2A) (7–9), protein phosphatase 1 (PP1) (10, 11), protein phosphatase 1D (PPM1D; or Wip1) (12–14), and cell division cycle 14 (Cdc14) (15), have previously been implicated in the regulation of p53 phosphorylation via their phosphatase activities. There are 18 serine and threonine residues in p53 (16) that are phosphorylated in response to genotoxic and nongenotoxic stress (16–18), PP2A and dephosphorylate Ser-46 (9), and Wip1 may dephosphorylate Ser-15 (12, 13) of p53. Ser-46 is responsible for the induction of apoptotic genes and PTEN, whereas Ser-15 is associated with cell cycle arrest and Mdm2 (19). However, p53 mutants lacking some of these phosphorylation sites do not seem to overtly alter p53 function (20, 21), suggesting that the

\textsuperscript{a}The abbreviations used are: ATM, ataxia-telangiectasia mutated; ATR, ATM and Rad3-related; PP, protein phosphatase; Cdc, cell division cycle; PTEN, phosphatase and tensin homolog; DOX, doxorubicin; LDM, low-density mononuclear; KO, knockout; eGFP, enhanced GFP; MEF, mouse embryonic fibroblast; DNA-PK, DNA-dependent protein kinase; PP5ca, constitutively active form of PP5; co-IP, coimmunoprecipitation; GST, GST S-transferase; CHX, cycloheximide; SUMO, small ubiquitin-like modifier.

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This article contains Table S1.

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regulation of p53 phosphorylation is a highly complex event and may require other posttranslational modifications to achieve significant changes in biological activity (22).

Protein phosphatase 5 (PP5) is a serine/threonine phosphatase that contains a 34-amino-acid tetrapeptide repeat domain that is known to mediate protein–protein interactions and serves as an autoinhibitory domain for the phosphatase activity of PP5 (23). The catalytic domain of PP5 is similar to those of other protein phosphatases, such as PP1, PP2A, and PP2B (24). PP5 is ubiquitously expressed and is believed to have multiple functions in several intracellular signaling networks, including cell cycle regulation (25) and cellular stress responses (26). Interestingly, several recent clinical studies have shown that PP5 is up-regulated in breast cancers, and the human PP5 gene resides at a chromosomal region that is frequently amplified in osteosarcoma patients (27). In a xenograft model, a 2-fold increase in PP5 protein levels significantly enhanced the growth rate of estrogen-dependent tumors (28). These studies suggest an important role for PP5 in tumorigenesis. Additionally, PP5 has been shown to be associated with several genotoxic stress–induced protein complex members, such as apoptosis signal-regulating kinase 1 (29, 30), the DNA-dependent Ser/Thr protein kinase DNA-PKcs (31), ATM/ATR (32–34), Raf1 (35), and the glucocorticoid receptor (36–38). Using a gene knockdown approach in cultured cells, Honkanen and coworkers (25, 39, 40) demonstrated that PP5 can act as a negative regulator of p53 function; however, the molecular mechanism by which PP5 regulates p53 function has not been closely analyzed.

Previously, we generated pp5-deficient mice and showed that pp5-deficient cells possess altered ATM-mediated signaling and function (32). However, this regulation was likely indirect as ATM is not known to be a PP5 substrate (41). In the current study, we identified p53 as a strong candidate substrate of PP5. Biochemical analyses demonstrated that PP5 was able to directly interact with p53 and dephosphorylate this protein at multiple Ser/Thr sites both in vitro and in vivo. PP5 protein levels were significantly elevated in pp5-deficient cells and tissues and down-regulated in pp5-overexpressing cells compared with the levels in the normal controls. Subsequent analysis further demonstrated that hyperphosphorylation of p53 in pp5-deficient cells stabilized p53. In addition, we also identified two conserved putative p53-binding sites in the pp5 promoter region. Chromatin immunoprecipitation (ChIP) and luciferase assays confirmed that p53 is a potent transcriptional repressor of PP5. Compound pp5−/−/pp5−/− mutant mice exhibited significantly longer lifespans and later onset of tumorigenesis than mice that were double heterozygous for both genes (pp5+/−/pp5+/−). Collectively, our findings reveal a unique regulatory interplay between PP5 and p53, which likely constitutes a novel positive feedback mechanism involved in the cellular response to stress.

Results

Mice deficient in PP5 exhibit increased sensitivity to genotoxic stress and up-regulation of p53 protein levels

Our previous studies suggested that pp5-deficient cells exhibit defects in the G2/M cell cycle checkpoint in response to DNA damage (32). To further explore the mechanism of this phenomenon, we tested the biological response of the genotoxic reagent doxorubicin (DOX) using bone marrow low-density mononuclear (LDM) cells isolated from pp5-deficient (knockout (KO)) and sex-matched wildtype (WT) littermate mice. DOX was shown to be able to induce generation of superoxide and hydroxyl radicals, which can cause DNA oxidative damage, which subsequently leads to eventual cell cycle arrest and cellular apoptosis (42, 43). Using flow cytometry analysis, we found that fewer than 5% of the KO or WT LDM cells were apoptotic in the absence of DOX treatment (Fig. 1A). However, the percentage of apoptotic KO cells increased dramatically after DOX treatment (0.05 or 0.1 μg/ml; 12 h) compared with similarly treated WT cells (21.26 ± 0.49 and 41.94 ± 8.64% in PP5 KO cells versus 10.76 ± 1.22 and 25.21 ± 4.68% in WT cells, p < 0.001) (Fig. 1A). The result indicated that pp5-deficient bone marrow LDM cells are hypersensitive to DOX treatment. p53 is regarded as a critical sensor for cell stress and an important modulator of apoptosis in response to a range of stimuli (44, 45). The stress-induced apoptosis in hematopoietic progenitors is p53-dependent (46). To test whether PP5 is a regulator of p53 function, we examined whether p53 expression was altered in pp5-deficient LDM cells by Western blot analysis, which showed that PP5 mutant bone marrow cells had significantly higher p53 levels than the littermate controls (Fig. 1D). Next, we tested whether the increased cellular apoptosis observed in pp5-deficient bone marrow cells in response to DOX treatment was pp5-dependent. RNA interference (RNAi) was used to knock down p53 by transducing WT and KO primary bone marrow cells with a retrovirus (pMSCV-eGFP/sh-p53) or a control virus carrying only eGFP (pMSCV-eGFP) (47). Following transduction, cells were sorted for eGFP to enrich the transduced cells and treated with DOX. Real-time quantitative RT-PCR was used to confirm the p53 levels in the control and p53 RNAi–treated cells along with the levels of the p53 downstream regulators MDM2 and p21. In both pp5-deficient and WT cells, p53 levels were efficiently reduced by RNAi, and this was accompanied by a reduction in MDM2 and p21 expression (Fig. 1C). We found that the increase in cell death in pp5-deficient cells in response to DOX treatment was diminished following p53 knockdown (Fig. 1B), further supporting the hypothesis that p53 mediates the biological function of PP5 in stress-induced apoptosis.

To understand the physiological relevance of the interplay between PP5 and p53, an in vivo disease model of DOX-mediated cardiotoxicity was used as DOX treatment increases heart p53 protein levels and leads to cardiomyocyte atrophy (i.e. reduced size of cardiomyocytes) (48). A loss-of-function p53 mutant effectively blocked this DOX-mediated cardiomyocyte atrophy (48). To test whether PP5 mutant hearts were hypersensitive to DOX, 20 mg/kg DOX was administered to 2-month-old pp5-deficient and WT littermate control mice for 7 days, and saline was used as a control treatment (48). Based on the minimal diameters of the cardiomyocytes, we determined that the baseline size of the cardiomyocytes in pp5-deficient hearts was significantly smaller when compared with that in the WT controls (11.68 ± 0.54 μm in pp5-deficient cardiomyocytes versus 12.57 ± 0.28 μm in WT cardiomyocytes, n = 400
randomly selected cardiomyocytes/three animal hearts, \( p < 0.01 \), and this was associated with a higher level of p53 in \( pp5^- \)-deficient hearts than in WT hearts (Fig. 2, A and B). As predicted, DOX treatment was able to cause a significant reduction in the minimal diameter of the cardiomyocytes in \( pp5^- \)-deficient hearts compared with that in WT hearts (9.7 ± 1.51% reduction in \( pp5^- \)-deficient cardiomyocytes versus 7.7 ± 1.23% reduction in WT cardiomyocytes, \( n = 400 \) randomly selected cardiomyocytes/three animal hearts, \( p < 0.05 \); Fig. 1, E and F). This finding confirms the importance of PP5 in p53-mediated physiology.

In addition to the elevated p53 expression levels in bone marrow (Fig. 1D), we also evaluated p53 protein levels in adult and embryonic tissues of WT and \( pp5^- \)-deficient mice, including the thymus, spleen, heart, and liver. As observed in bone marrow cells, p53 levels were significantly elevated in all the \( pp5^- \)-deficient tissues examined (Fig. 2A). Similarly, mouse embryonic fibroblasts (MEFs) isolated from \( pp5^- \)-deficient embryos (E12.5) had significantly elevated levels of p53 compared with control MEFs isolated from WT littermates (Fig. 2C). The phospho-p53 Ser-15 levels were also elevated (Fig. 2C).

To determine whether the increased levels of p53 in \( pp5^- \)-deficient mice enhanced the transcriptional activity, the expression levels of the downstream targets MDM2 (49, 50), PTEN (51), and p21 (52) were analyzed in thymus and heart samples via Western blotting. \( pp5^- \)-deficient tissues consistently exhibited higher levels of these p53 target genes than the WT controls (Fig. 2B). Moreover, the p21, Mdm2, and Pten mRNA levels in \( pp5^- \)-deficient MEFs were up-regulated compared with the levels in the WT control cells (Fig. 2, E, F, and G). However, the p53 mRNA levels were not altered in \( pp5^- \)-deficient cells (Fig. 2D), indicating that the elevation in p53 protein levels was likely due to the enhanced p53 stability in \( pp5^- \)-deficient cells.
Figure 2. p53 levels and activity were elevated in pp5-deficient mice. A, Western blot analyses show that p53 expression was significantly elevated in the selected pp5-deficient tissues, thymus, thymus, heart, and liver. B, expression of the p53 target genes MDM2, PTEN, and p21 was increased in pp5-deficient mouse thymus (Thy) and heart. C, total and phospho-p53 (Ser-15) were detected in WT pp5−/− MEF cells using anti-p53 and anti-Ser-15 p53 antibodies. D, real-time quantitative RT-PCR indicated that the level of p53 mRNA remained unchanged in mutant MEF cells compared with the levels in the WT controls. p21 (E), Mdm2 (F), and Pten (G) mRNA levels were also increased in pp5-deficient MEF cells, as shown by real-time quantitative RT-PCR analysis of WT and pp5−/− cells. Values are expressed as the means ± S.D. (error bars) from three independent experiments. ** represents $p < 0.01$, and *** represents $p < 0.001$. 

PP5 deficiency reduces tumorigenesis in heterozygous p53+/− mice

Given that pp5-deficient mice exhibit increased p53 protein levels, we hypothesized that the PP5–p53 interplay may play a role in tumorigenesis. To test this hypothesis, we generated the compound mutant mice p53+/−pp5−/− and p53+/−pp5+/+. Both strains had one copy of p53, resulting in a moderate level of p53 expression, and one or no copies of pp5, resulting in moderate to no expression of PP5. Western blotting was used to confirm the significantly higher expression levels of p53 in p53+/−pp5−/− mice than in the pp5+/−p53+/− littersmates (Fig. 3A) in the thymus, brain, and liver. The lifespan of p53+/−pp5−/− mice was significantly extended (Fig. 3B) with an increase in median lifespan observed from days 257 to 379. Both the p53+/−pp5+/+ and p53+/−pp5−/− double mutant mice presented malignant tumors; however, delayed tumor onset was observed in p53+/−pp5−/− mice compared with p53+/−pp5+/+ mice (Table 1). Tumor types were analyzed and are listed in Table S1. Therefore, the data further suggest that PP5 is a functional regulator of p53.

PP5 interacts with p53 and dephosphorylates phospho-p53 at multiple Ser/Thr sites

Phosphorylation of p53 was shown to increase the stability of the p53 protein (16). To test the hypothesis that PP5 acts as a serine/threonine phosphatase that dephosphorylates p53, HEK293T cell lysate was immunoprecipitated with an anti-p53 antibody, and the immunoprecipitates were incubated in the absence or presence of purified PP5 for 30 min at 30 °C. Incubation was followed by Western blot analysis using antibodies that recognized specific phosphorylated residues in p53 (i.e. Ser-9, Ser-15, Ser-20, Ser-37, and Ser-46). As shown in Fig. 4A, we found that PP5 was able to dephosphorylate multiple sites in p53 in vitro, namely Ser-15, Ser-20, and Ser-37. Interestingly, we observed dephosphorylation at Ser-46, which is a site that is important for the induction of apoptotic genes (53). In contrast, the phosphatase Wip1 can dephosphorylate p53 at only Ser-15 (54). It was previously demonstrated that DNA-dependent protein kinase (DNA-PK) phosphorylates p53 at Ser-15 (55). We directly tested whether PP5 could dephosphorylate DNA-PK–induced phospho-p53 Ser-15. DNA-PK was first incubated with purified p53 in kinase reaction buffer for 30 min. After heating at 65 °C to inactivate the kinase, purified recombinant PP5 was added to the reaction. The level of p53 phosphorylation was monitored by Western blot analysis using anti-phospho-p53 (Ser-15) and anti-p53 antibodies. As shown in Fig. 4B, phospho-p53 Ser-15 levels were dramatically reduced following the addition of PP5. To further test the observed phenomenon in vivo, we transfected a constitutively active form of PP5 (PP5ca), which harbored a 13-amino-acid truncation at the C terminus, into WT and pp5−/− MEF cells. PP5ca expression reduced the relative levels of total p53 and phospho-p53 in WT and pp5−/− cells (Fig. 4C). Furthermore, Western blot analysis of total p53 and phospho-p53 was performed in transgenic mice overexpressing PP5ca. Our data demonstrated that both total p53 and phospho-p53 levels were dramatically

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Figure 3. Phosphorylation of p53 is increased in pp5-deficient tissues. A, immunoblot of thymus, brain, and liver whole extracts using an anti-p53 and anti-phospho-p53 (Ser-15) antibody. B, expression of the p53 target genes MDM2, PTEN, and p21 was increased in pp5-deficient mouse thymus (Thy) and heart. C, total and phospho-p53 (Ser-15) were detected in WT pp5−/− MEF cells using anti-p53 and anti-Ser-15 p53 antibodies. D, real-time quantitative RT-PCR indicated that the level of p53 mRNA remained unchanged in mutant MEF cells compared with the levels in the WT controls. p21 (E), Mdm2 (F), and Pten (G) mRNA levels were also increased in pp5-deficient MEF cells, as shown by real-time quantitative RT-PCR analysis of WT and pp5−/− cells. Values are expressed as the means ± S.D. (error bars) from three independent experiments. ** represents $p < 0.01$, and *** represents $p < 0.001$.
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Figure 3. PP5 deficiency enhances survival in p53<sup>+/−</sup> mice. A, Western blot analyses showed that p53 expression was significantly elevated in pp5<sup>−/−</sup> p53<sup>+/−</sup> tissues relative to the expression in p5<sup>−/−</sup> p5<sup>+/−</sup> tissues. B, survival curves of p5<sup>−/−</sup> p5<sup>+/−</sup> (n = 15) and pp5<sup>−/−</sup> p5<sup>+/−</sup> (n = 21) mouse cohorts.

Table 1

| Genotype | p53<sup>−/−</sup> | pp5<sup>−/−</sup> | p53<sup>−/−</sup> | pp5<sup>+/−</sup> |
|----------|-----------------|-----------------|-----------------|-----------------|
| Number of mice | 15 | 21 | 14 | 18 |
| Number of animals analyzed by necropsy | 11 (78%) | 10 (55%) | 12 (86%) | 15 (88%) |

It is known that p53 protein levels are primarily regulated via various posttranslational modifications. Phosphorylation of p53 was thought to be associated with p53 stability. The half-life of p53 in WT cells was expected, the absence of PP5 significantly prolonged the half-life of p53. As shown in Fig. 3, the expression of PP5 was negatively regulated by p53. The mRNA expression level of pp5 was significantly higher in the p53 mutant liver than in the WT liver. Furthermore, when p53 was transfected into p53-deficient MEFs, the PP5 expression level was greatly reduced.

To determine whether p53 can regulate PP5 expression, we compared p53 expression levels between WT and p53-deficient MEFs by Western blotting. Our data showed that PP5 expression was up-regulated in p53-deficient MEFs compared with the expression in WT cells (Fig. 5B). We also compared PP5 expression between WT and p53-deficient MEFs using quantitative RT-PCR. As shown in Fig. 5F, pp5 mRNA expression was significantly higher in the p53 mutant liver than in the WT liver (Fig. 5F), which was consistent with the protein level measurements (Fig. 5E). Furthermore, when p53 was transfected into p53-deficient MEFs, the PP5 expression level was greatly reduced (Fig. 5D). Taken together, these data suggest that p53 acts as a negative regulator of PP5.

**PPS expression is negatively regulated by p53**

PP5 has been shown to be up-regulated in tumor cells (26, 59). In an effort to determine the underlying mechanism, a survey of potential transcriptional binding sites for the human and mouse pp5 promoters was performed. Two conserved p53-binding sites were identified within the pp5 promoter region (Fig. 5A). p53 binds specifically to a consensus DNA sequence consisting of two copies of the 10-bp motif 5′-(A/G)(A/G)(A/G)(T/A)(T/A)(G/T/C)(T/C/T/C)-3′ separated by 0–13 bp (60). This sequence has been observed in many p53 regulatory genes, including p21/Whf1, Mdm2, Bax, Gadd45, and PcnA. To determine whether p53 can regulate PP5 expression, we compared PP5 expression levels between WT and p53-deficient MEFs by Western blotting. Our data showed that PP5 expression was up-regulated in p53-deficient MEFs compared with the expression in WT cells (Fig. 5B). We also compared PP5 expression between WT and p53-deficient MEFs using quantitative RT-PCR. As shown in Fig. 5F, pp5 mRNA expression was significantly higher in the p53 mutant liver than in the WT liver (Fig. 5F), which was consistent with the protein level measurements (Fig. 5E). Furthermore, when p53 was transfected into p53-deficient MEFs, the PP5 expression level was greatly reduced (Fig. 5D). Taken together, these data suggest that p53 acts as a negative regulator of PP5.

To determine whether p53 directly binds to the consensus sites identified above in the pp5 promoter region, we performed a ChIP assay using p53-overexpressing H1299 cells (a well-known human cell line that is deficient in endogenous p53). As shown in Fig. 5G, p53-specific ChIP bands were readily amplified in anti-p53 immune complexes, and both consensus pp5-binding sites were detected by PCR analysis. To further confirm the importance of the pp5 promoter region, 1.5 kb of the mouse pp5 promoter was subcloned into the promoter-less luciferase expression vector pGL3 (Promega). In parallel, H1299 cells were cotransfected with pPP5-promoter-Luc plus GLA-Renilla and either pcDNA-WT-p53, the pcDNA vector control, or pcDNA-mutant-p53 (codon 173, GTG→GTA), which was mutated in the DNA-binding domain. As shown in Fig. 5H, WT p53 strongly repressed luciferase activity, but mutant p53 (codon 173, GTG→GTA) and the pcDNA vector control did not exhibit similar repression. To further understand whether this activity depended on p53-binding sites (Fig. 5A), a mutant.
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**Promoter-luciferase construct was generated that lacked the p53-binding sites.** The luciferase assay showed that the repressive regulatory ability of p53 was lost after removal of the p53-binding sites from the pp5 promoter (Fig. 5H). These data suggested that p53 binds directly to the pp5 promoter and functions as a negative regulator of pp5 transcription.

**Discussion**

In this study, we report the discovery of a novel PP5–p53 interplay with implications for p53-mediated apoptosis and tumorigenesis. Both bone marrow LDM cells and cardiomyocytes from pp5-deficient mice were studied and found to be hypersensitive to DOX treatment. Interestingly, p53 expression was significantly elevated in various tissues of pp5-deficient mice, indicating the importance of PP5 in disease development.

Using an siRNA interference approach to knock down p53, we showed, for the first time, that p53 mediates the biological function of PP5 in stress-induced apoptosis. Consistent with elevated p53 expression in pp5-deficient mice, the p53 downstream genes *p21* and *Pten* were significantly up-regulated in terms of mRNA and protein expression levels in pp5-deficient mice compared with the levels in the WT controls (Fig. 2, B–G). Notably, a comparison of survival led to a promising result: the p53−/−/H11002/pp5−/−/H11002 mice survived 122 days longer, on average, than the p53−/−/H11001/pp5−/−/H11001 mice (Fig. 3B). Using *in vitro* biochemical analyses, we demonstrated that PP5 directly interacts with p53 and dephosphorylates phospho-p53 at multiple Ser/Thr sites. Furthermore, consensus p53-binding sites were identified within the pp5 promoter region by bioinformatics analysis, and the role of these sites in PP5 repression was confirmed via a

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**Figure 4.** PP5 directly dephosphorylates phospho-p53 at multiple sites and interacts with p53. A, p53 immunoprecipitates were incubated in the presence (−) or absence (+) of purified PP5 for 30 min at 30 °C followed by Western blot analysis with antibodies against phosphorylated p53 in different tissues. B, purified PP5 dephosphorylated p53 at Ser-15 *in vitro*. Lane 1 represents the untreated p53 control. Following treatment with DNA-PK and ATP, p53 (lane 2) was incubated with purified PP5 (lane 3). Anti-Ser-15 p53 and anti-p53 monoclonal antibodies were used for Western blotting analyses. C, in constitutively active PP5-overexpressing MEF cells, the phospho-p53 levels were significantly lower than the levels in the WT and PP5 mutant cells. D, the total p53 and phospho-p53 levels were significantly decreased in the PP5ca transgenic mice. The interaction of p53 and PP5 was examined by co-IP (E) and GST pulldown assays (F). G, PP5 KO increased the half-life of p53. Four hours after 4-gray ionizing radiation treatment, the WT and PP5 KO MEF cells were treated with 200 g/ml CHX for the indicated times. Lysates were prepared and analyzed by Western blotting for p53 using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. H, levels of p53 were quantified by densitometry, and optical density was plotted as the percentage of p53 protein remaining. The p53 band intensity was normalized to the glyceraldehyde-3-phosphate dehydrogenase band intensity and then to the t = 0 controls. IB, Immunoblotting; Tg, transgenic.
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ChIP assay (Fig. 5). Taken together, our data indicate that these interactions represent a positive feedback loop between p53 and PP5 in response to cellular and genotoxic stress.

p53 has been found to have multiple functions in the maintenance of genome integrity, cellular apoptosis, senescence, cell cycle control, metabolism, stem cell reprogramming, and autophagy. As p53 is a stress-induced transcription factor, we explored the interactions between PP5 and p53 during genotoxic stress by examining p53-induced apoptosis in pp5-deficient mice. The results indicated that high p53 levels are accompanied by hypersensitivity to DOX treatment in pp5-deficient mice. Different p53 functions are regulated by posttranslational modifications, including acetylation, phosphorylation, methylation, ubiquitination, SUMOylation, and O-GlcNAcylation (21). Phosphorylation of Ser or Thr sites is critical for the regulation of the degradation, stabilization, and transcriptional activity of p53 (5). Our in vitro phosphorylation experiments indicated that PP5 dephosphorylates phospho-p53 at not only Ser-15 but also some additional sites in vitro (Fig. 4A) in contrast to Wip1, which exclusively targets Ser-15 (54). Previous studies demonstrated via suppression of pp5 expression using an antisense pp5 oligonucleotide that PP5 appears to function as a negative regulator of p53 at Ser-15, which is consistent with our findings. However, they did not observe a decrease in the phosphorylation level of p53 following overexpression of PP5 (25). Several in vitro studies have demonstrated that PP5 usually exhibits low phosphatase activity under normal conditions (23), which is likely due to the interaction between the small autoinhibitory domain at the C terminus (residues 490–499) and the tetrafunctional repeat domain. This interaction prevents potential substrate access to the PP5 catalytic domain (62). It has been shown that PP5 can be activated ~10-fold in vitro via a 13-amino-acid C-terminal truncation (62, 63). Therefore, the discrepancies between previous findings and the current study likely result from our usage of PP5ca rather than WT PP5. In our experimental system, PP5ca maintained the total p53 levels at a significantly low value relative to the levels in the control. These results were obtained both in vitro and in vivo (Fig. 4, C and D).

p53-deficient mice are developmentally viable, but these mice exhibit reduced survival due to the development of various tumors within 10 months of age, including lymphomas, sarcomas, carcinoma, and osteosarcoma (64). p53 heterozygous mice also develop tumors but at a later age. Approximately 50% of heterozygous p53 mice develop tumors by 18 months of age. By 2 years, >95% of heterozygous mice die of tumors in contrast to a death rate of only ~20% in their WT littermates (65). Our studies on pp5-deficient mice have revealed the role of PP5 in regulating the functions of p53, especially stability and activity. We hypothesized that PP5 could functionally regulate p53-mediated tumorigenesis. To test this hypothesis, we produced pp5+/− pp5−/− and pp5+/− pp5−/− double mutant mice and measured survival. Reduced p53 levels in pp5+/− mice can lead to increased genomic instability, which increases the likelihood of the development of somatic p53-null cells (66). We found that the p53 levels in the pp5+/− pp5−/− mice were enhanced enough to either prevent or reduce tumorigenesis, consequently increasing longevity by an average of 122 days (Fig. 3B).

Beyond its function as a transcriptional activator, p53 also functions as a transcriptional repressor (67). We observed that PP5 expression was up-regulated in MEFs and in tissues of pp5-deficient mice (Fig. 5, B and C). It has been reported that there are two distinct types of repression mediated by p53: those that require consensus p53-binding elements and those that do not require such elements. Polo-like kinase 1 (68) and Cdc25c (69) are critical mitotic checkpoint genes that are subject to p53-mediated repression. Similar to Polo-like kinase 1 and Cdc25c, the pp5 promoter contains a consensus p53-binding element. Our experiment using promoter-driven luciferase and ChIP assays demonstrated that p53 directly binds to the pp5 promoter, resulting in PP5 repression (Fig. 5, G and H).

In summary, our work shows that PP5 is a protein phosphatase that is capable of directly regulating p53 phosphorylation, stability, and function and that the expression of PP5 is negatively regulated by p53 (Fig. 6). This novel regulatory interplay may provide the feedback necessary for altering the response of p53 in response to cellular stress.

Materials and methods

Mice

The present study used pp5 KO and littermate WT mice maintained in a C57BL/6 background. Adult mice received two intraperitoneal injections of DOX (10 mg/kg) or vehicle (saline) at 3-day intervals and were euthanized 7 days after the initial injection. p53+/− male mice in a C57BL/6 background were crossed with pp5+/− females to generate p53+/− pp5+/−, and then crossing of p53+/− pp5−/− males and females was performed to generate p53+/− pp5−/− and p53+/− pp5−/− mice. Male p53+/− pp5+/− and p53+/− pp5−/− mice were kept under a standard light/dark regimen (12-h light/12-h dark) for further studies. All animal experiments were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” and were approved by the Animal Care and Research Advisory Committee of the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences, and the Indiana University School of Medicine.

Cell lines and culture

MEF cells were obtained from WT and pp5-deficient (KO) embryos at day 13.5 of gestation (70). H1299 and p53−/− MEFs...
were gifts from Dr. Hu Lu. Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

**In vitro phosphorylation and dephosphorylation of p53**

Purified recombinant p53 was obtained from Dr. Lindsey Mayo. DNA-PK was purchased from Promega (catalog number P91V581). PP5 cDNA was cloned into pET21 with BamHI/EcoRI, and the GST fusion protein was expressed, purified, and cleaved as described using B-PER GST Fusion Protein Purification kits (Thermo Fisher Scientific). p53 was phosphorylated in vitro by DNA-PK according to the manufacturer's instructions. After heating at 65 °C to inactivate the kinase, purified recombinant PP5ca was added to the reaction with phosphatase assay buffer (50 mM Tris, 4 mM MnCl₂, 4 mM MgCl₂, 1 mM EGTA, 0.1% 2-mercaptoethanol, pH 7.6). The level of phosphorylated p53 was assessed by Western blot analysis using anti-phospho-p53 (Ser-15) and anti-p53 antibodies. The p53 immunoprecipitates were incubated in the absence (−) or presence (+) of purified PP5 for 30 min at 30 °C followed by Western blot analysis with antibodies against different p53 phosphorylation sites.

**Coimmunoprecipitation and in vivo GST pulldown**

Approximately 1 mg of total protein obtained from cotransfected H1299 cells was coimmunoprecipitated using a Pierce co-IP kit (catalog number 26149) following the manufacturer's instructions. Anti-p53 (DO-1, sc-126, Santa Cruz Biotechnology, Inc.) and anti-IgG (A7007, Beyotime, China) were used for antibody immobilization and to pull down p53. Anti-PP5 antibody (H-170, sc-67039, Santa Cruz Biotechnology, Inc.) was used to detect the interaction between p53 and PP5. For the in vivo GST pulldown assay, equal amounts of GST and GST-pp5 expression vectors were transfected into HEK293T cells as indicated. Cell lysates were incubated with GSH-Sepharose beads, and the amount of endogenous p53 pulled down was assayed by Western blotting using a p53 antibody (DO-1).

**p53 half-life**

p53 protein half-life studies were performed as described by McVean et al. (71). WT and pp5 deletion MEF cells were treated with ionizing radiation for 4 h. CHX (2 μg/ml) was then added to inhibit further protein synthesis. Cells were harvested in radioimmune precipitation assay buffer 15, 30, 45, and 60 min after CHX treatment. Aliquots containing 100 μg of total protein were analyzed by Western blotting.

**Bone marrow cell culture and retroviral transduction**

Bone marrow LDM cells from pp5 KO and WT mice were purified using a Ficoll gradient as described previously (72). After 24 h of prestimulation, the cells were treated with 0, 0.01, and 0.1 μg/ml DOX (Sigma) for 12 h. The treated cells were stained with annexin V–allophycocyanin (BD Pharmingen) followed by flow cytometry according to the manufacturer’s instructions. Ecotropic retroviral supernatants (pMSCV and pMSCV-p53) were prepared using Eco-Phoenix packaging cells. Bone marrow cells were then transferred and sorted as described previously (61) and treated with DOX as described above.

**Histology**

Hearts were harvested, cryoprotected in 30% sucrose, and sectioned at 10 μm using standard techniques. To quantitate minimal cardiomyocyte fiber diameters, images from Sirius Red/Fast Green–stained sections were captured, digitized, and analyzed with NIH ImageJ software as described previously (48). At least 400 randomly selected cardiomyocytes from each animal were analyzed.

**Gel electrophoresis and Western blotting**

Samples were resolved on denaturing SDS gels. Transfer of the samples to Immobilon-P® membranes and immunoblotting were performed as described previously (26). Primary antibodies were used to detect the following targets: p53, p53 Ser-15, p53 Ser-20, PP5, p21, and PTEN. The blots were then incubated with the appropriate peroxidase-conjugated secondary antibodies followed by detection using enhanced chemiluminescence.

**Quantitative RT-PCR**

Total RNA was isolated from mouse tissues or cells using TRIzol (Invitrogen). First-strand cDNA was synthesized by...
using the Transcriptor First-Strand cDNA Synthesis kit (Roche Applied Science) using 1 μg of RNA as a template according to the manufacturer's instructions. Real-time PCR was performed using a LightCycler 480 with LightCycler 480 SYBR Green I Master Mix (Roche Applied Science). The relative expression levels of the PCR products were normalized to Rpl7.

ChIP and luciferase assay

ChIP assays were performed using the ChIP Assay kit (Millipore). The lysates were immunoprecipitated with either rabbit IgG or an anti-p53 antibody (DO-1). The primers used in the PCRs are shown in Fig. 5A. The mouse pp5 promoter containing the p53-binding site was amplified from genomic DNA with the following primers: pr F, 5'-TAATGGTACCCTCTGGAAGCTTCC-3'; pr R, 5'-ATCGACATCTAACATATCCACCCAGCCAGCAC-3'. The amplified fragment was then cloned into the pGL3-luciferase vector (Promega) between the KpnI and BglII sites. Luciferase activity was analyzed 48 h after transfection, and transfection efficiency was normalized with a Renilla expression vector.

Table 2

| Primer name | Sequence |
|-------------|----------|
| Mouse Rpl7_F | CAGAGTTCGAGTCCAGGCTCTG |
| Mouse Rpl7_R | TCCCTTCTGAGTCCACATCC |
| Mouse Mdm2_F | CTCTGATGTCAGGATGAGGAGCC |
| Mouse Mdm2_R | GAGATGGATTCATCTGGGAGAGG |
| Mouse Pten_F | TCCACTTGGGATGACGTCG |
| Mouse Pten_R | CACCGAGATCCGGAAGGAA |
| Mouse p21_F | CAGCTGCTGGCTATTGCTCC |
| Mouse p21_R | CCGGCGGGATTGAGGAGG |
| Mouse ps3_F | GAGATTCCTTGGCCAGACTCC |
| Mouse ps3_R | GTTATCCACGACCCAGCTTC |
| Human RPL7_F | GCGCAAGAATCAGGACGCCCTGAGAA |
| Human RPL7_R | GCCATCCCTGACCGAGGAA |
| Human PPS_F | TCTAAGGTTGGCAGGAGTTG |
| Human PPS_R | TGAACAGGCTTCCGCTTACG |
| Human p21_F | TGCTTCTACATCTGGGAGG |
| Human p21_R | ATCCGCGGTCTTGGAGG |
| Human MDM2_F | GAGACGTCTGGCAGCTTGTTGG |
| Human MDM2_R | AGCTCCGAGTATCTGGGAGG |
| Human ps3_F | GCCCTCTCACCAGCTGACAC |
| Human ps3_R | TGTTTTGAGCAGCTGTCG |

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cultured human keratinocytes exposed to bis-(2-chloroethyl) sulfide. J. Appl. Toxicol. 15, 477–482 CrossRef Medline

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