Down-regulation of Wild-type p53-induced Phosphatase 1 (Wip1) Plays a Critical Role in Regulating Several p53-dependent Functions in Premature Senescent Tumor Cells

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Background: Wip1 is a phosphatase involved in DNA-damage response.

Results: Wip1 expression is down-regulated in premature senescent cancer cells. Failure to down-regulate Wip1 expression results in cell death and polyploidy.

Conclusion: Wip1 down-regulation is important for maintenance of permanent cell cycle arrest in premature senescent tumor cells.

Significance: These findings improve our understanding of the mechanism by which Wip1 promotes tumor progression.

Premature or drug-induced senescence is a major cellular response to chemotherapy in solid tumors. The senescent phenotype develops slowly and is associated with chronic DNA damage response. We found that expression of wild-type p53-induced phosphatase 1 (Wip1) is markedly down-regulated during persistent DNA damage and after drug release during the acquisition of the senescent phenotype in carcinoma cells. We demonstrate that down-regulation of Wip1 is required for maintenance of permanent G2 arrest. In fact, we show that forced expression of Wip1 in premature senescent tumor cells induces inappropriate re-initiation of mitosis, uncontrolled polyploid progression, and cell death by mitotic failure. Most of the effects of Wip1 may be attributed to its ability to dephosphorylate p53 at Ser15 and to inhibit DNA damage response. However, we also uncover a regulatory pathway whereby suppression of p53 Ser15 phosphorylation is associated with enhanced phosphorylation at Ser46, increased p53 protein levels, and induction of Noxa expression. On the whole, our data indicate that down-regulation of Wip1 expression during premature senescence plays a pivotal role in regulating several p53-dependent aspects of the senescent phenotype.

Cellular senescence was initially described as a growth-arrest program that limits the lifespan of normal mammalian cells (1). Additional work has demonstrated that, apart from aging, various physiologic stresses induce a rapid onset of cell senescence, often referred to as “stress-induced” or “premature senescence.” Such stresses include oncogene activation, DNA-damaging agents, and several different stimuli (2). Although cancer cells bypass both replicative and oncogene-induced senescence, some senescence pathways remain intact and can be reactivated by expression of critical regulators (3, 4). In particular, treatment with sublethal concentrations of conventional DNA-damaging anticancer agents readily induces premature senescence in cancer cells (5). Premature senescence is a major cellular response to chemotherapy and radiotherapy in solid tumors (6, 7), and an intact senescence pathway critically contributes to the success of chemotherapy (8). Hence, the induction of senescence has been proposed as a potential strategy for therapeutic intervention in cancer (9, 10). Senescent cells remain viable, metabolically active, and acquire a complex phenotype, including distinctive morphological alterations (flat and enlarged morphology), the expression of acidic β-galactosidase, and a specific increase in the secretion of cytokines, chemokines, and other factors, which has been termed the “senescence-associated secretory phenotype” or SASP (11, 12). p53 has a well defined role in establishment and maintenance of growth arrest during senescence (13, 14). More recently, it has also been reported that p53 negatively modulates the SASP (11). As a result, cells lacking p53 secrete higher levels of several SASP components (11). PPM1D or Wip1 (wild-type p53-induced phosphatase) belongs to the Ser/Thr PP2C family of phosphatases (15). Members of this evolutionarily conserved family are frequently involved in the regulation of cellular stress responses (15, 16). Wip1 is both a direct transcriptional target of p53 and an important negative regulator of p53, thus creating a negative regulatory feedback loop (17, 18). Wip1 also dephosphorylates several other DNA damage-responsive proteins, such as ATM, ATR, Chk1, Chk2, and p38MAPK (19).

In this work we investigated the role of Wip1 phosphatase in premature senescence in cancer cells. Here we show that Wip1...
levels are decreased in chemotherapy-induced senescence. We demonstrate that down-regulation of Wip1 is required for maintenance of permanent G1 arrest. Accordingly, forced expression of Wip1 in premature senescent tumor cells suppresses phosphorylation of p53 at serine 15 and induces inappropriate re-initiation of mitosis, uncontrolled polyploid progression, and cell death by mitotic failure. Interestingly, dephosphorylation of p53 at Ser\(^{15}\) is associated with enhanced phosphorylation at Ser\(^{46}\) and induction of Noxa gene expression. Finally, premature senescent cells forced to express Wip1 develop an amplified SASP.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Biological Reagents**—A549 cells were obtained from American Type Culture Collection and cultured according to its instructions. MCF-7 cells were cultured in DMEM. All media were supplemented with 10% fetal bovine serum. The cell culture media and reagents were purchased from Invitrogen. Doxorubicin (Merck) was dissolved in sterile water. Bleomycin sulfate (Merck) was dissolved in DMSO. Pifithrin-α (Sigma) was dissolved in DMSO. z-VAD-fmk (Merck) was dissolved in DMSO.

**Induction of Premature Senescence, Senescence-associated β-Galactosidase Activity, and Micronuclei Detection**—Unless otherwise stated, senescence was induced by treating cells with the DNA-damaging agents doxorubicin (200 nM for MCF-7 cells and 600 nM for A549 cells) for 72 h as previously described (20). Because forced Wip1 expression leads to death of premature senescent cells, all the experiments were conducted from 1 to 7 days after drug release. Staining for acidic β-galactosidase was performed as previously described (21). Micronuclei were detected in senescent cells by phase-contrast microscopy. For each sample at least 300 nuclei were analyzed.

**Lentiviral Constructs and Infection of Cells**—The Wip1 and the Wip1-FLAG plasmids were generously provided by Prof. Galit Lahav and Prof. Xiongbin Lu, respectively. Wip1-FLAG cDNA was subcloned into pWPT lentiviral vector at BamHI site. The construct was sequenced to confirm correct DNA sequence and orientation.

Subconfluent 293T lentivirus packaging cells were cotransfected with either pWPT-GFP or Wip1-FLAG-pWPT and pMD2G and pCMV-R8.91 by calcium phosphate precipitation. After 24 h the medium was changed, and supernatant was harvested after 48 and 72 h. Lentiviral supernatant, cleared of cell debris, was concentrated by centrifugation for 90 min at 23,000 rpm.

For transduction, MCF7 and A549 cells were plated on 12-well plates and infected with lentiviruses in the presence of 10% fetal bovine serum and 8 μg/ml Polybrene (hexadimethrine bromide; Sigma). After 48 h of incubation, infection efficiency was determined by analyzing GFP expression by flow cytometry.

**Treatment with siRNAs**—MCF7 cells were treated with 200 nM doxorubicin for 72 h, extensively washed, and released in drug-free media. Cells were transfected with either control siRNA (Dharmacon, ON-TARGETplus Non-targeting pool) or with Wip1 siRNA (Sigma MISSION® esiRNA #EHU009271) using Oligofectamine (Invitrogen) according to the manufacturer's protocol.

**Cell Cycle Analysis**—Cells were fixed with 70% ethanol in PBS and routinely kept at −20 °C overnight. Cells were washed twice with PBS, resuspended in PBS, 40 μg/ml propidium iodide (Sigma), and 50 μg/ml RNase DNase-free (Roche Applied Science), and incubated at room temperature for 20 min. Cells were analyzed using a CyAn ADP Flow Cytometer (Beckman Coulter, Inc., Milano, Italy) and Summit Software.

**Immunofluorescence Microscopy**—Detection of γ-H2AX, phosphorylated ATM (Ser\(^{1981}\)) and phosphorylated Chk2 (Thr\(^{68}\)) was performed as previously described (20). The anti-γ-H2AX antibodies (JBW301) were purchased from Upstate Biotechnology (Milton Keynes, UK), and anti-phospho-ATM (Ser\(^{1981}\)) and anti-phospho-Chk2 (Thr\(^{68}\)) were from Cell Signaling (Beverly, MA). DAPl (4,6-diamidino-2-phenylindole) was purchased from Sigma.

**Cell Viability**—Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron coupling reagent (phenazine methosulfate) according to the manufacturer’s instructions (Promega). For the MTS assay, 5 × 10\(^{3}\) senescent cells were seeded in triplicate into a 96-well plate and analyzed for cell viability at the indicated times.

**Measurement of Mitochondrial Membrane Potential**—Measurements of mitochondrial membrane potential were performed by staining cells with tetramethylrhodamine-ethyl ester (TMRE) (Invitrogen). A TMRE stock was prepared at a concentration of 10 μM in DMSO and stored at −20 °C. Cells were loaded with TMRE by incubating cells in media containing 50 nM TMRE for 30 min at 37 °C. After two washes, cells were kept at 4 °C and analyzed immediately using a CyAn ADP Flow Cytometer (Beckman Coulter) and Summit Software.

**Live/Dead Assay**—LIVE/DEAD Fixable Green Dead Cell Stains (Invitrogen) was used according to the manufacturer’s instructions to measure the viability of the cells at different times after induction of senescence. Cells were analyzed using a CyAn ADP Flow Cytometer (Beckman Coulter) and Summit Software.

**Measurement of Apoptosis and Necrosis**—Apoptosis was detected by flow cytometry using PE Annexin V Apoptosis Detection Kit I (BD Biosciences). Briefly, cells were double-stained with annexin V-PE and 7-AAD following the manufacturer’s instructions. Early apoptosis is defined by annexin V-positive/7-AAD negative staining, and late apoptosis/necrosis is defined by annexin V-positive/7-AAD positive staining. Cells were analyzed using a CyAn ADP Flow Cytometer (Beckman Coulter) and Summit Software.

**Western Blot Analysis**—Total cell proteins preparations were obtained lysing cells in a lysis buffer containing 50 mM TrisCl, pH 7.4, 2 mM EDTA, 0.1% Triton X-100, 1% Nonidet-P40, 100 mM NaCl, 1 μg/ml aprotinin, and 170 μg/ml phenylmethylsulfonyl-fluoride (Sigma). Protein concentration was routinely measured by the Bio-Rad protein assay. Western blot analysis was carried out according to standard procedures using ECL detection from GE Healthcare. The anti-Wip1 (A300-664A) antibody was purchased from Bethyl Laboratories (Bethyl Laboratories Inc., Montgomery, TX). The anti-Wip1 (H300), anti-
FIGURE 1. Expression of Wip1 in senescent tumor cells. A, A549 cells were treated with 200 nM doxorubicin for 72 h, extensively washed, and replated in drug-free media. Expression of Wip1 protein was analyzed at indicated time points during drug treatment and during the development of the senescent phenotype. Filters were stripped and reprobed with anti-actin antibody as a loading control. B, MCF7 cells were treated with 200 nM doxorubicin for 72 h, extensively washed, and replated in drug-free media. Expression of Wip1 protein was analyzed at the indicated time points during drug treatment and during the development of the senescent phenotype. Filters were stripped and reprobed with anti-actin antibody as a loading control. C, shown is expression of Wip1 protein in proliferating and senescent MCF7 cells transduced with GFP or FLAG-Wip1. Filters were stripped and reprobed with anti-actin antibody as a loading control. "*" indicates a nonspecific cross-reactive band. D, MCF7, MWIP1, and MGFP cells were treated with either doxorubicin (1 μM, 2 h) or with bleomycin (20 μg/ml, 2 h), and phosphorylation of p53 at Ser15 was analyzed by Western blot. Filters were stripped and reprobed with anti-p53 and anti-Wip1 antibodies. α-Tubulin was used as a loading control. E, shown is expression of Wip1 protein in proliferating and senescent A549 cells transduced with GFP or FLAG-Wip1. Filters were stripped and reprobed with anti-actin antibody as a loading control. "*" indicates a nonspecific cross-reactive band. F, A549, AWIP1, and AGFP cells were treated with either doxorubicin (doxo, 1 μM, 2 h) or with bleomycin (bleo, 20 μg/ml, 2 h), and phosphorylation of p53 at Ser15 was analyzed by Western blot. Filters were stripped and reprobed with anti-p53 and anti-Wip1 antibodies. α-Tubulin was used as a loading control. prol, proliferating cells; sen, senescent cells.

RESULTS

Decrease in Wip1 Protein Level during Premature Senescence—To investigate the role of Wip1 phosphatase in modulating premature senescence in tumor cells, we treated the lung adenocarcinoma cell line A549 with doxorubicin for 72 h as previously described (20). We analyzed Wip1 protein level during drug treatment and thereafter during the development of the senescent phenotype over several days after the initial DNA damage. An increase in Wip1 protein was readily detectable 24 h after the start of the treatment, then followed by a gradual decrease starting from 48 h treatment (Fig. 1A). Wip1 is amplified in various human cancers (22–25). To investigate the regulation of Wip1 in the context of gene amplification, we induced premature senescence in the breast cancer cell line MCF7. MCF7 cells carry an amplified PPM1D/Wip1 gene and...
overexpress the phosphatase (22). In line with the data obtained in the A549 cell lines, treatment with doxorubicin resulted in increased Wip1 protein at 24 h, then followed by a progressive decrease under the base line (Fig. 1B). The phosphatase was almost undetectable in fully senescent MCF7 cells (Fig. 1B, 15 days). These data were confirmed by immunoblot analysis with a different anti-Wip1 antibody (supplemental Fig. 1). Hence, Wip1 is down-regulated during persistent DNA damage and after drug release during the acquisition of the senescent phenotype. This down-regulation is detected also in cells with deregulated Wip1 gene expression resulting from gene amplification.

**Effects of Wip1 Overexpression in Premature Senescent Tumor Cells**—To investigate the functional role of Wip1 down-regulation in premature senescence, we studied the effects of forced expression of Wip1. MCF7 and A549 cells were infected with lentiviruses encoding either for GFP or for FLAG-tagged Wip1 (Fig. 1C: MGFP and MWIP1, derived from MCF7 cells; Fig. 1E: AGFP and AWIP1, derived from A549). Because Wip1 dephosphorylates several DNA damage response proteins and acts as a negative regulator of p53 (17), we first confirmed the ability of FLAG-Wip1 to dephosphorylate p53 at Ser15 in cells treated with either doxorubicin or bleomycin. As shown in Fig. 1, D and F, expression of FLAG-Wip1 attenuated DNA damage-induced p53 phosphorylation at Ser15 in both MCF7 and A549 cells. In addition, expression of FLAG-Wip1 also reduced constitutive p53 Ser15 phosphorylation in senescent cells (supplemental Fig. 4A).

Next, we induced senescence in all cells by treatment with doxorubicin. A characterization of the senescent cells is illustrated in supplemental Fig. 1. Both GFP- and FLAG-Wip1-expressing cells acquired a fully senescent phenotype, with morphological alterations and acidic β-galactosidase staining typical of premature senescent cells (supplemental Fig. 1B). In addition, both GFP- and FLAG-Wip1 cells developed a senescence-associated secretory phenotype (data not shown). It is important to note that, different from endogenous Wip1, elevated levels of transduced FLAG-Wip1 were maintained in both cell lines after induction of senescence (Fig. 1, C and E). Hence, Wip1 does not prevent drug-induced senescence. However, a gradual loss of cell viability during senescence, assessed by MTT assay, was observed in MWIP1 cells as compared with control cells (MGFP and MCF7 cells) (Fig. 2A). The progressive loss of viability was further confirmed in both senescent MWIP1 and AWIP1 cells by means of a live/dead assay (Fig. 2, B and C).

Because senescent cells accumulate persistent DNA damage foci (26) and because Wip1 directly dephosphorylates several DNA damage response proteins (27), we next analyzed phospho-ATM (Ser1981) and γ-H2AX foci formation in FLAG-Wip1-expressing lines. As shown in supplemental Fig. 2, whereas persistent P-ATM and γ-H2AX foci were readily detected in senescent MCF-7 and A549 cells, no foci were observed in FLAG-Wip1-expressing cells.

**Wip1 Overexpression Overrides G2 Phase Arrest and Promotes Mitotic Cell Death**—Recent studies revealed a critical role for Wip1 in conferring G2 checkpoint recovery competence by counteracting p53-dependent transcriptional repres-
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A

B

FIGURE 3. Cell cycle distribution in senescent carcinoma cells. A, shown are representative flow cytometric data. Cell cycle distribution in proliferating and senescent cells (4 days after release) was analyzed by flow cytometry after propidium iodide (PI) staining. The percentage of cells in G1, S, G2/M, or sub-G1 phase is given. B, shown are representative flow cytometric data. Senescent cells (4 days after release) were stained with propidium iodide and analyzed by flow cytometry. The percentage of cells with >4N DNA content is given.

sion of mitotic regulators (28). Because senescent tumor cells mainly arrest in the G2 phase of the cell cycle (29) (Fig. 3A), we analyzed the expression of cyclin B1 in proliferating and senescent MGFP, MWIP1, AGFP, and AWIP1 cells. As shown in Fig. 4A, acquisition of the senescent phenotype is accompanied by suppression of cyclin B1 protein in GFP control cells. In contrast, elevated levels of cyclin B1 were detected in FLAG-Wip1-expressing cells. In addition, a clear correlation between cyclin B1 levels and pRb phosphorylation status was detected (Fig. 4A). Interestingly, Wip1 protein levels appeared to progressively decline in the senescent cells (Fig. 4A and data not shown), an effect likely attributable to a selection against Wip1-expressing senescent cells. Notably, under the conditions used for routine propagation of the cells, i.e. in the absence of senescence induction, cells maintain a relatively stable level of FLAG-Wip1 expression.

Data in Fig. 4A raise the possibility that down-regulation of Wip1 in premature senescence may be required to inhibit inappropriate cell cycle re-entry, with unrepaired DNA damage. Indeed, flow cytometric analyses of histone H3 phosphorylation at serine 10 revealed that a significant subset of FLAG-Wip1 senescent cells progress from G2 into mitosis (Fig. 4B). Furthermore, a significant polyploid cell fraction, characterized by a >4N DNA content, appeared in senescent cells forced to express Wip1 (Fig. 3B). We employed 5-bromo-2-deoxyuridine (BrdU) incorporation as a measure of the numbers of cells engaging in DNA synthesis. As shown in Fig. 4C, a significant fraction of G2-arrested cells, forced to express Wip1, enter S phase, likely resulting in polyploidy.

To determine the role of endogenously overexpressed Wip1 in the regulation of p53 functions in premature senescent tumor cells, we silenced Wip1 expression in senescent MCF7 cells using RNA interference. As shown in Fig. 5A, treatment with Wip1-specific siRNA reduced the expression of Wip1 in senescent cells at all concentrations tested. Inhibition of Wip1 increased the phosphorylation of p53 at serine 15 in a dose-dependent manner. Next, we analyzed the ability of endogenous Wip1 to counteract the p53-dependent prolonged G2 arrest. Senescent MCF7 cells were transfected with control siRNA or Wip1 siRNA and analyzed for the expression of cyclin B1 and PARP-1 cleavage. As shown in Fig. 5B, Wip1 knockdown resulted in down-regulation of cyclin B1 and PARP-1 cleavage (Fig. 5B). More importantly, silencing of endogenous Wip1 resulted in a significant decrease in the frequency of polyploid cells (8N) (Fig. 5C).

On the whole, these data suggest that, by suppressing both ATM (supplemental Fig. 2) and p53 phosphorylation, Wip1 induces inappropriate re-initiation of mitosis from G2 phase, uncontrolled polyploid progression, and cell death by mitotic failure.

Mitotic catastrophe is characterized by the occurrence of aberrant mitosis, resulting in the accumulation of large cells with several micronuclei (30). Accordingly, we observed a significant increase in the number of micronucleated senescent cells when Wip1 was constitutively expressed (Fig. 6). Cells undergoing mitotic catastrophe can die by either apoptosis or necrosis (31). Hence, we induced senescence in all cells and analyzed cell death by annexin V/7-AAD staining. As shown in Fig. 7, in both cell lines, forced expression of Wip1 induced a significant increase in both early apoptotic (annexin V-positive, 7-AAD-negative) and late apoptotic/necrotic cells (annexin V-positive, 7-AAD-positive). Treatment with the pan-caspase inhibitor z-VAD-fmk significantly reduced apoptosis in both MWFIP1 and AWIP1 cells. Interestingly, z-VAD-FMK also partially inhibited late apoptosis in MWIP1 senescent cells (Fig. 7A). Finally, Western blot analyses showed both caspase-9 activation and PARP-1 cleavage in senescent MWFIP1 cells (supplemental Fig. 3). In contrast, we did not observe activation of either caspase-9 or caspase-8 or caspase-3 or PARP cleavage in senescent AWIP1 cells undergoing mitotic catastrophe (data not shown). These data suggest that both apoptosis and necrosis are induced in senescent cells forced to express Wip1.

Wip1 Overexpression Affects p53 Phosphorylation Status—To get more insight into the ability of Wip1 to cause cell death in premature senescent tumor cells, we examined p53 and
p21<sup>CIP1</sup> protein levels in proliferating and senescent cells. Both AWIP1 and MWIP1 senescent cells showed increased levels of p53 and reduced amounts of p21<sup>CIP1</sup> proteins as compared with control cells (Fig. 8A and supplemental Fig. 4B). In addition, p53 was found to accumulate not only in the nucleus but also in the cytoplasm of FLAG-Wip1-expressing cells (supplemental Fig. 4, C and D). As expected, accumulated p53 protein was not phosphorylated at serine 15 in Wip1-overexpressing cells (supplemental Fig. 4E and data not shown). Transcriptional activation of p53 is modulated by post-translational modifications. Phosphorylation on Ser<sup>15</sup> by ATM and ATR is a central event during DNA damage and has been shown to mediate both p53 stabilization and activation (for review, see Ref. 32). However, studies using mouse mutants with substitutions of Ser<sup>15</sup> suggest that this residue is not essential for p53 activation (33, 34). Because both MWIP1 and AWIP1 senescent cells showed
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A

FIGURE 5. Effects of Wip1 silencing on cell cycle regulators and polyplody. A, MCF-7 cells were treated with 200 nM doxorubicin for 72 h, extensively washed, and released in drug-free media. Cells were transfected with either control siRNA (50 nM) or with Wip1 siRNA (50 or 100 nM). Expression of Wip1 protein was analyzed at 48 h or 72 h after transfection. Filters were stripped and reprobed with anti-phospho-Ser15 p53, anti-p53, and anti-cyclin B1 antibodies. * indicates nonspecific cross-reactive band. ** indicates the presence of constitutively active Wip1. B, MCF-7 cells were treated with 200 nM doxorubicin for 72 h, extensively washed, and released in drug-free media. Cells were transfected with either control siRNA or with Wip1 siRNA (50 nM each). Expression of Wip1 protein was analyzed at 48 h or 72 h after transfection. Filters were stripped and reprobed with anti-phospho-Ser46 p53, anti-p53, and anti-cyclin B1 antibodies. * indicates nonspecific cross-reactive band. ** indicates the presence of constitutively active Wip1. C, MCF-7 cells were treated with 200 nM doxorubicin for 72 h, extensively washed, and released in drug-free media. Cells were transfected with either control siRNA or with Wip1 siRNA (50 nM each). Senescent cells were stained with propidium iodide and analyzed by flow cytometry at the indicated time points after transfection. Data represent the percentage of cells with 8N DNA content as compared with controls. Data are the mean ± S.E. from three independent experiments. Statistical analysis was by unpaired Student’s t test: *, p ≤ 0.03; **, p ≤ 0.005; ***, p ≤ 0.0001.

increased levels of p53 and the accumulated p53 protein was not phosphorylated at Ser15, we decided to further investigate p53 post-translational modifications in FLAG-Wip1-expressing cells. First, we used phage λ-phosphatase to analyze the phosphorylation status of p53 in senescent A549, AGFP, and AWIP1 cells. Both in controls (A549 and AGFP) and in AWIP1 cells, a pronounced phosphatase-dependent shift in p53 electrophoretic mobility was observed, indicating that in premature senescent tumor cells p53 is phosphorylated, even in the presence of constitutively active FLAG-Wip1 (supplemental Fig. 4F). Phosphorylation of p53 at Ser46 regulates the ability of p53 to induce apoptosis (35, 36). Hence, we analyzed p53 phosphorylation at Ser46 during the development of the senescent phenotype in AGFP and AWIP1 cells. It was found that phosphorylation at Ser46 is selectively induced in senescent cells forced to express Wip1 (Fig. 8B).

To investigate if the increased p53 protein in these cells was functional and to investigate the role of Ser46 phosphorylation in regulating the transcription of pro-apoptotic genes in premature senescent tumor cells, we analyzed the expression of a number of proapoptotic p53 target genes (such as Puma, Noxa, Perp, Pig3, Apaf1). Of the genes tested, Noxa (PMAIP1) was significantly induced in both senescent MWIP1 and AWIP1 cells, as compared with control cells (Fig. 8C and D, and data not shown). During apoptosis, Noxa induces mitochondrial dysfunction (37). Hence, we measured the changes in mitochondrial membrane potential (∆Ψm) by staining with TMRE. As shown in Fig. 9A and B, forced Wip1 expression induces premature senescent MCF7 cells to undergo mitochondrial depolarization. The same results were obtained in A549 cells (data not shown). In addition, we examined the effects of Pifithrin-α (PFT-α), an inhibitor of p53 transactivation (38), on Wip1-dependent up-regulation of Noxa. We first confirmed the ability of PFT-α to prevent the up-regulation of p21CIP1 in proliferating cells treated with doxorubicin (supplemental Fig. 5A). Next, to specifically inhibit pro-apoptotic p53 (i.e. phospho-Ser46 p53), we treated deep senescent AWIP1 and MWIP1 cells (percentage of cells showing reduced mitochondrial membrane potential >25%) with PFT-α and analyzed Noxa expression by real time PCR. As shown in Fig. 9C, PFT-α significantly
suppressed Noxa expression and attenuated mitochondrial depolarization in Wip1-overexpressing cells (Fig. 9, D and E). Notably, PFT-α did not affect polyploidization in Wip1-expressing cells (supplemental Fig. 5B).

These data indicate that constitutive Wip1 expression during premature senescence counteracts p53-dependent repression of mitotic genes and compromises permanent G2 arrest, likely through dephosphorylation of p53 at Ser15. However, phosphorylation of different residues, such as Ser46, still allows p53 pro-apoptotic signaling.

**DISCUSSION**

In this manuscript we present evidence pointing to Wip1 as a critical regulator of cell fate after induction of premature senescence. We demonstrate that Wip1 expression is down-regulated during the acquisition of the senescent phenotype and that this down-regulation is required for a permanent arrest in the cell cycle. Indeed, ectopic expression of Wip1 in premature senescent cells results in an inappropriate re-entry in the cell cycle (via cyclin B1), polyploid progression, or cell death. These effects seem to be mediated by different p53 phosphorylation (decreased Ser15 and increased Ser46 phosphorylation) and induction of the proapoptotic gene Noxa.

Wip1 belongs to the conserved PP2C phosphatase family (15), whose members are frequently involved in the regulation of cellular stress responses (15, 16). Accordingly, expression of Wip1 is readily induced in response to DNA damage in a p53-dependent manner (15). Wip1 dephosphorylates several DNA damage-responsive proteins, such as ATM, ATR, Chk1, Chk2, and p38MAPK (19). Furthermore, Wip1 also dephosphorylates p53 at Ser15, thus attenuating the DNA damage response (DDR) (41). Cytotoxic drugs as well as ionizing radiation are able to induce senescence in tumor cells expressing wild-type p53 both in vitro and in vivo (5–7). The senescent phenotype does not
develop after transient DNA damage but develops slowly, over several days, and is associated with chronic DDR (11). We show that when the DNA damage signal lasts for a long time, i.e., during a persistent DNA damage that induces premature senescence in tumor cells, Wip1 protein is reduced. Interestingly, persistent DNA damage results in Wip1 down-regulation also in MCF-7 cells, which overexpress the phosphatase as a consequence of gene amplification. Repression of Wip1 protein during chronic DDR and in pathological aging has been recently demonstrated in a mouse model of progeria (42). In this model, suppression of Wip1 has been related to miR-29 up-regulation (42). We are currently investigating if a similar mechanism is also responsible for Wip1 down-regulation in our experimental system.

To investigate the biological significance of Wip1 down-regulation in premature senescence, we studied the effects of forced expression of Wip1. Wip1 protein levels do not prevent drug-induced senescence; in fact both AWIP1 and MWIP1 cells develop a full senescent phenotype after treatment with doxorubicin. However, our results demonstrate that down-regulation of Wip1 is required for maintenance of permanent G2 arrest in premature senescent tumor cells. Forced expression of
Wip1 suppresses DDR and induces inappropriate re-initiation of mitosis, as demonstrated by analyses of histone H3 phosphorylation. Because Wip1 dephosphorylates most DDR proteins, i.e. ATM, Chk2 (data not shown), and γ-H2AX, cells enter mitosis with unrepaired DNA and undergo cell death likely by mitotic failure. This effect appears to be tumor cell-specific. In fact, although normal senescent cells arrest with a G1 DNA content, premature senescent tumor cells characteristically arrest in the G2 phase of the cell cycle (29), likely due to defective G1 checkpoint of cancer cells (43). Accordingly, it has been shown that introduction of Wip1 in normal human mesenchymal stem cells (G1 arrested) allows bypass of senescence and extends cellular life span (44).

Mitotic catastrophe has recently been defined as a mechanism that senses mitotic failure and drives the cells to death (45). In tumor cells, mitotic catastrophe has been associated with compromised G2/M checkpoint signaling (46). In our experimental system, forced expression of Wip1 induces dephosphorylation of p53 at Ser15 and the inappropriate expression of mitotic regulators, which prevents G2 arrest and triggers mitotic catastrophe. In particular, we show that the induction of premature senescence is associated with suppression of cyclin B1 protein in control cells, whereas elevated levels of cyclin B1 are detected in cells expressing Wip1. Increased cyclin B1 expression in senescent tumor cells correlates with pRb phosphorylation. These observations are consistent with
the essential role played by Wip1 in regulating p53-dependent transcriptional repression of mitotic regulators (28). Notably, silencing of endogenously overexpressed Wip1 in premature senescent MCF-7 cells results in increased p53 phosphorylation at Ser\textsuperscript{15} and decreased cyclin B1 expression. Hence, although expression of Wip1 during acute DNA damage is critical to successfully recover from the arrest (28), down-regulation of Wip1 is required during persistent DNA damage to establish a permanent G\textsubscript{2}/M cell cycle arrest in tumor cells. Cells that undergo mitotic catastrophe can die by two separate mechanisms, i.e., apoptosis or necrosis, and the cell type appears to determine the final pattern of cell death (30). In our study, forced expression of Wip1 in premature senescent tumor cells induces both apoptosis and necrosis, as estimated by annexin V/7-AAD staining. In MWIP1 senescent cells undergoing mitotic catastrophe caspase-9 was clearly activated (and PARP-1 cleaved). Although we did not observe activation of either caspase-9 or caspase-8 or caspase-3 in senescent AWIP1.
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Our study shows that deregulated expression of Wip1 during a persistent DNA damage, which induces premature senescence in tumor cells, results in the accumulation of polyploid cells. This activity provides potential mechanisms by which Wip1 may promote tumor progression.

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