Senescence Sensitivity of Breast Cancer Cells Is Defined by Positive Feedback Loop between CIP2A and E2F1


Tumor Suppression

- p53 activity
- \( \downarrow \)
- p21
- \( \downarrow \)
- E2F1
- \( \downarrow \)
- CIP2A

Tumor Progression

- p53 activity
- \( \downarrow \)
- p21
- \( \downarrow \)
- E2F1
- \( \rightarrow \)
- CIP2A
ABSTRACT

Senescence induction contributes to cancer therapy responses and is crucial for p53-mediated tumor suppression. However, whether p53 inactivation actively suppresses senescence induction has been unclear. Here, we show that E2F1 overexpression, due to p53 or p21 inactivation, promotes expression of human oncoprotein CIP2A, which in turn, by inhibiting PP2A activity, increases stabilizing serine 364 phosphorylation of E2F1. Several lines of evidence show that increased activity of E2F1-CIP2A feedback renders breast cancer cells resistant to senescence induction. Importantly, mammary tumorigenesis is impaired in a CIP2A-deficient mouse model, and CIP2A-deficient tumors display markers of senescence induction. Moreover, high CIP2A expression predicts for poor prognosis in a subgroup of patients with breast cancer treated with senescence-inducing chemotherapy. Together, these results implicate the E2F1-CIP2A feedback loop as a key determinant of breast cancer cell sensitivity to senescence induction. This feedback loop also constitutes a promising prosenescence target for therapy of cancers with an inactivated p53–p21 pathway.

SIGNIFICANCE: It has been recently realized that most currently used chemotherapies exert their therapeutic effect at least partly by induction of terminal cell arrest, senescence. However, the mechanisms by which cell-intrinsic senescence sensitivity is determined are poorly understood. Results of this study identify the E2F1-CIP2A positive feedback loop as a key determinant of breast cancer cell sensitivity to senescence and growth arrest induction. Our data also indicate that this newly characterized interplay between 2 frequently overexpressed oncoproteins constitutes a promising prosenescence target for therapy of cancers with inactivated p53 and p21. Finally, these results may also facilitate novel stratification strategies for selection of patients to receive senescence-inducing cancer therapies. Cancer Discov; 3(2); 182–97. © 2013 AACR.

INTRODUCTION

Cellular senescence functions as a barrier that normal cells have to overcome to transform into cancer cells (1). Accordingly, analysis of several types of premalignant tumors, most notably benign skin nevi, has revealed the existence of senescent pretumorigenic cells (1, 2). The functional relevance of spontaneous senescence induction in preventing tumor initiation and progression has been shown by several recent mouse studies (3–5).

Notably, although traditionally considered as apoptosis-inducing agents, most of the currently used chemotherapies exert their therapeutic effect at least partly by senescence induction (6, 7). Similarly, evidence is accumulating that despite the essential role of the tumor suppressor p53 in mediating apoptosis induction by genotoxic stimuli and chemotherapies, its in vivo tumor suppressor activity is not dependent on apoptosis, but rather on senescence induction (8–11). However, p53 function is inactivated in the majority of human cancers, and p53 inactivation correlates with poor patient survival in several cancer types including breast cancer (12). Traditionally, resistance of p53-mutant cells to chemotherapy has been linked to defective checkpoint function of p53 (13). However, we cannot exclude the possibility that, in addition to defective checkpoint activity, p53 inhibition actively promotes a mechanism or mechanisms that confer cancer cells’ general resistance to chemotherapy-induced senescence. In addition to mutations, p53 is known to be inactivated in cancer cells by enhanced proteolytic degradation driven by the ubiquitin ligases MDM2 and MDMX (14). Although therapeutic strategies to activate senescence via inhibition of MDM2/MDMX-p53 interactions have been under intense research lately (14), because of p53 mutations, they are unlikely to be efficient in a large fraction of human...
tumors. Therefore, the need to identify novel mechanisms that promote senescence resistance and tumor progression downstream of inactivated p53 is urgent. Identification of such mechanisms would not only provide novel insights into senescence regulation but could also facilitate development of novel prosenescence therapeutic strategies for cancers harboring inactivated p53 (6, 7).

E2F1 is an oncogenic transcription factor that is overexpressed in various human cancer types (15). Recent studies have indicated that E2F1’s classic function in transcriptional activation of S-phase-associated genes only partially explains its oncogenic activity (15, 16). Its transcriptional activity is negatively regulated by p53 through p21-mediated regulation of retinoblastoma (Rb) protein phosphorylation (15, 16), but expression and activity of E2F1 are also regulated directly by phosphorylation, independently of Rb (16, 17). The p53 reactivation by small-molecule activator Nutlin-3 inhibits protein expression of E2F1 and induces senescence-like growth arrest (18). Accordingly, knockdown of E2F1 expression also induces cellular senescence in p53-deficient cancer cells and blocks tumor growth (19–21). However, the mechanisms by which E2F1 prevents senescence induction in p53-deficient cells are currently unclear.

The human oncoprotein cancerous inhibitor of PP2A (CIP2A) is overexpressed in 65% to 90% of tissues from patients in almost all human cancer types studied thus far, and its expression correlates with cancer progression in a large variety of human malignancies (Supplementary Table S1; refs. 22–25). Even though CIP2A protein expression correlates with proliferation in human cancers (22–25), expression of CIP2A is not regulated by cell-cycle activity (24). Overexpressed CIP2A transforms immortalized cells of either human or mouse origin (23, 26), whereas its depletion by RNA interference (RNAi) inhibits anchorage-independent growth of several types of tumor cells (22–26). CIP2A’s tumor-promoting role has been shown by several xenograft studies (22, 23, 25, 26), but the genetic evidence that it contributes to tumor progression is yet lacking. CIP2A’s oncogenic function has been mostly linked to its ability to prevent proteolytic degradation of MYC by promoting its serine 62 phosphorylation (23, 24, 27, 28). As CIP2A overexpression is one of the most frequent alterations in human cancers (Supplementary Table S1), identification of novel mechanisms that regulate CIP2A, and oncogenic targets that could explain its significant correlation with human cancer progression, would be of general interest.

Here, we show that CIP2A is a direct transcriptional target of E2F1 and that CIP2A overexpression increases expression of E2F1, phosphorylated at serine 364. The positive feedback loop between these 2 human oncoproteins is stimulated by p53 inactivation, and is critical for inhibition of senescence induction in human breast cancer cells. Moreover, our results strongly indicate that the E2F1–CIP2A positive feedback loop plays a role in the resistance toward senescence-inducing chemotherapy in patients with breast cancer. Furthermore, we provide the first genetic evidence for CIP2A’s role in promoting breast cancer progression. Our data also indicate that this newly identified oncogenic mechanism is a potential prosenescence target for treatment of cancers with inactivated p53.

RESULTS
CIP2A Expression Is Associated with p53 Expression and Adverse Prognostic Factors in Human Breast Cancer

High CIP2A mRNA expression positively correlates with the presence of p53 mutation in human breast cancer samples (22). To confirm that p53 inactivation in breast cancer cells correlates with CIP2A protein expression, a series of unselected human breast cancers were stained for CIP2A and p53 protein expression, by using a p53 antibody that we have recently shown to be indicative of p53 mutation (29). Of the 1228 cancers investigated, 46% were positive for CIP2A (Supplementary Fig. S1A and S1B), and CIP2A expression significantly correlated with high p53 immunopositivity (Fig. 1A and B). However, despite statistical correlation between high p53 immunopositivity and increased CIP2A protein expression (Fig. 1B), this analysis identified tumors in which CIP2A was highly expressed even in the absence of p53 immunopositivity. It is possible that in these cases CIP2A overexpression is due to high expression of MYC or ETS1 transcription factors, both shown recently to stimulate CIP2A expression in human cancer cells (24, 30). Moreover, CIP2A expression correlated significantly with several markers of aggressive disease, such as a high Ki-67 proliferation index, a large tumor size, and a low histologic grade of differentiation (Fig. 1B and Supplementary Fig. S1C and S1D).

Wild-type p53 Downregulates CIP2A Expression

To study whether wild-type (WT) p53 negatively regulates CIP2A expression, p53 expression was inhibited by siRNA in cultured mouse embryonic fibroblasts (MEF), and CIP2A expression was subsequently studied by Western blotting. As shown in Fig. 1C, inhibition of p53 expression in MEFs by 2 different siRNA sequences resulted in robust induction of CIP2A protein expression. Moreover, reactivation of WT p53 in MCF-7 human breast cancer cells with small-molecule inhibitors of the Mdm2–p53 interaction, Nutlin-3 (31) or RITA (32), inhibited CIP2A expression at both the mRNA and protein levels (Fig. 1D and E and Supplementary Fig. S2A and S2B). To confirm that CIP2A downregulation by Nutlin-3 is dependent on WT p53 function, we treated MDA-MB-231 human breast cancer cells, harboring inactive mutant p53, with Nutlin-3. Nutlin-3 treatment had no effect on either p21 or CIP2A protein expression in MDA-MB-231 cells (Fig. 1F). However, when WT p53 was introduced to these cells, CIP2A protein expression was inhibited in a concentration-dependent manner (Fig. 1G). To further confirm that CIP2A expression is regulated by a p53-dependent mechanism, we treated isogenic WT and p53−/− HCT116 human colorectal cancer cells with the p53-activating chemotherapy doxorubicin. In contrast to WT cells, p53−/− HCT116 cells were resistant to doxorubicin-induced inhibition of CIP2A mRNA expression (Fig. 1H). In addition to in vitro models, we analyzed CIP2A expression in lymphoma tissue derived from a transgenic Eβ-Myc mouse model carrying tamoxifen-inducible p53 (33). As shown in Fig. 1I and J, in vivo restoration of p53 function resulted in inhibition of CIP2A expression in lymphoma tissue, thus confirming that p53 also negatively regulates oncoprotein CIP2A expression in vivo.
in addition to the experimental data above, bioinformatic analysis of a recently published CIP2A-regulated gene signature (34) with Ingenuity Transcription Factor Analysis software, which reads transcription factor activities, showed that the transcriptional response to CIP2A knockdown mimicked most significantly the situation in which p53 is activated (Fig. 1K and Table S2). These results together identify CIP2A as a novel in vivo target of WT p53 activity and indicate that p53-mediated CIP2A downregulation functionally contributes to the p53 response.

E2F1 Uregulates CIP2A Expression Downstream of Inactivated p53

To study whether p53 regulates CIP2A expression at the transcriptional level, MCF-7 cells transfected with a CIP2A promoter luciferase construct containing the 1802 bp upstream promoter fragment (30) were treated with Nutlin-3 or RITA. The p53 reactivation either of these compounds inhibited the activity of the CIP2A promoter but not the activity of the EGF receptor (EGFR) promoter (35) that was used as a control (Fig. 2A and

**Figure 1.** WT p53 negatively regulates CIP2A expression. A, representative immunohistochemical stainings of CIP2A and p53 expression in human breast cancer tumors. B, CIP2A expression positively correlates with p53 expression and with proliferation marker Ki-67 in human breast tumors (n = 1228). P value calculated by χ² test. C, Western blot analysis of CIP2A expression in MEFs 48 h after transfection with scrambled (SCR) or 2 different p53 siRNAs (p53.1 and p53.2). D, Western blot analysis of CIP2A, p53, and p21 expression in MCF-7 cells treated with 2, 5, or 10 μmol/L of Nutlin-3 for 24 h. Shown is mean ± SEM of 2 independent experiments. E, representative result of 2 independent experiments with similar results. F, CIP2A protein expression in MDA-MB-231 human breast cancer cells harboring DNA binding–deficient p53 treated with 5 μmol/L of Nutlin-3 for 24 h. G, Western blot analysis of CIP2A expression in MDA-MB-231 cells 48 h after transfection with scrambled (SCR) or 2 different p53 siRNAs (p53.1 and p53.2). H, CIP2A protein expression in MDA-MB-231 cells harboring DNA binding–deficient p53 treated with 5 μmol/L of Nutlin-3 for 24 h.

**K** Pathway activity in CIP2A-depleted cells

<table>
<thead>
<tr>
<th>Transcription regulator</th>
<th>Predicted activation state</th>
<th>Regulation Z score</th>
<th>Z value</th>
<th>P value of overlap</th>
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<tr>
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<td>Inhibited</td>
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<tr>
<td>TP 53</td>
<td>Activated</td>
<td>2.204</td>
<td>2.32E-04</td>
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</table>
Supplementary Fig. S2C). Bioinformatic analysis of the ~1802 fragment of the CIP2A promoter revealed 2 putative p53 binding sites (Fig. 2B and Supplementary Fig. S2D). However, when a chromatin immunoprecipitation assay for p53 was conducted in doxorubicin-treated HCT-116 cells, we could not detect any enrichment for these 2 putative binding sites, although p53 clearly accumulated on Mdm2 or p21 promoters (Fig. 2C). In support of these results, p53 was found not to bind to the CIP2A promoter in chromatin immunoprecipitation sequencing (ChIP-Seq) analysis conducted with control or Nutlin-3–treated MCF-7 cells (data not shown; S. Aerts; personal communication). These results indicate that although p53 activity inhibits CIP2A gene transcription, CIP2A is not a direct target gene of p53.

The p53 downstream target, p21, regulates gene expression by inhibiting cyclin-dependent kinases (CDK), which in turn leads to dephosphorylation of Rb protein and consequent inhibition of an oncogenic transcription factor E2F1 (15, 16). We confirmed that Nutlin-3–induced CIP2A downregulation is associated with the activation of the above-described p21 cascade, leading also to the previously observed inhibition of
E2F1 protein expression (ref. 18; Fig. 2D). To study whether p21 induction is required for p53-mediated CIP2A downregulation, we used isogenic HCT-16 WT and p21−/− cells. In the unperturbed p21−/− cells, CIP2A expression was increased, as compared with that in WT cells (Fig. 2E). Interestingly, similar to p53−/− HCT-116 cells, p21−/− HCT-116 cells also were resistant to doxorubicin-induced CIP2A inhibition (Fig. 2E). Moreover, p21 expression by adenoviral transduction inhibited E2F1 and CIP2A expression in MDA-MB-231 cells harboring mutated p53 (Fig. 2F). Importantly, p21-elevated E2F1 inhibition was detected already at a 24-hour time point (1 day) and preceded downregulation of CIP2A protein expression (Fig. 2F). These results suggest that increased E2F1 expression may stimulate CIP2A expression in cells with inactive p53 and p21. In support of this hypothesis, CIP2A expression was inhibited in cells transfected with E2F1–targeting siRNA (Fig. 2G). Of note, CIP2A downregulation by E2F1 RNA interference (RNAi) is unlikely to be caused by general inhibition of cell-cycle activity, as CIP2A expression neither is sensitive to aphidicolin-elicted cell-cycle arrest nor is associated with serum-induced cell-cycle progression (24). Furthermore, conditional tetracycline-induced overexpression of E2F1 resulted in CIP2A upregulation at the mRNA level (Fig. 2H). To verify that CIP2A is a direct E2F1 target, we conducted E2F1 ChIP in cells transfected with an E2F1 expression construct. The E2F1 binding site at −378 to −361 in the −1802 fragment of CIP2A promoter was predicted by using Genomatix software. As shown in Fig. 2I, E2F1 antibody immunoprecipitation clearly enriched this putative CIP2A promoter E2F1 binding site from E2F1-overexpressing cells as compared with cells transfected with control vector or nonantibody controls. E2F1 binding to CIP2A promoter was further verified by ChIP-Seq analysis from MCF-7 cells by using the ENCODE database (Supplementary Fig. S2E).

Taken together, these results strongly implicate downregulation of CIP2A oncoprotein expression as a novel target mechanism for p53 tumor suppressor activity (Fig. 2J). Moreover, these results show that E2F1 stimulates CIP2A expression in cells with inactive p53 and p21 (Fig. 2J).

**Inhibition of CIP2A Expression Is a Prerequisite for p53-Mediated Senescence Induction**

In line with the indicated role for CIP2A as a p53 effector protein (Fig. 1K), CIP2A depletion by RNAi in MCF-7 cells mimicked p53-activated senescence, as characterized by increased senescence-associated β-galactoside (SA-β-gal) activity and flattened cell morphology in most of the cells (Fig. 3A). Induction of senescence was verified in CIP2A siRNA-transfected MCF-7 cells by increased expression of the p53-induced senescence marker decoy receptor 2 (DcR2; ref. 11; Fig. 3B). Importantly, CIP2A depletion also induced the appearance of the senescence phenotype in p53-mutant MDA-MB-231 cells (Fig. 3C), in which depletion of CIP2A causes long-term inhibition of xenograft tumor growth (22). Previously, we have shown that inhibition of CIP2A does not induce programmed cell death in HeLa cells (23). As hypothesized, stable expression of CIP2A did not reverse the obvious cell death phenotype in MCF-7 cells treated with RITA, a known inducer of p53-dependent cell death (Supplementary Fig. S2F and S2G; ref. 32). These results indicate that CIP2A downregulation is linked to p53-induced senescence.
CIP2A inhibits phosphatase activity of serine/threonine phosphatase PP2A (23, 42). Furthermore, inhibition of 2 regulatory B subunits of PP2A, B55α and B56β, rescues CIP2A depletion–induced effects on colony growth and gene expression (34). As a result, we hypothesized that PP2A holoenzymes consisting of either B55α or B56β subunits could be responsible for dephosphorylation of the serine 364 residue of E2F1 in cancer cells. In fact, inhibition of B55α, but not B56β, resulted in increased phosphorylation of serine 364 in E2F1 (Fig. 4F and Supplementary Fig. S3A). In addition, as with CIP2A overexpression, depletion of B55α rescued E2F1 protein downregulation induced by Nutlin-3 (Fig. 4G). Moreover, this effect was not observed with depletion of B56β (Fig. 4G). Taken together, these results suggest that the positive feedback mechanism from CIP2A to E2F1 is mediated by inhibition of the PP2A complex containing the B55α subunit.
Downregulation of E2F1 has been reported to induce senescence in a p53-independent manner and to prevent tumorigenesis (19–21). To show that loss of E2F1 results in induction of the senescent phenotype in the cell type studied, E2F1 expression was downregulated in MCF-7 cells by short hairpin RNA (shRNA; shE2F1). E2F1 depletion significantly increased the number of SA-β-gal-positive cells as compared with control cells expressing nontargeted shRNA (shNTC1) (Fig. 4H and I). Moreover, E2F1 downregulation either by Nutlin-3, or by E2F1 shRNA, mirrored their effectiveness in inducing the senescent phenotype, but Nutlin-3 could not further increase SA-β-gal positivity in E2F1-depleted cells (Fig. 4H and I). These results indicate that E2F1 downregulation is critical for senescence induction by Nutlin-3–elicited p53 reactivation.
Recent studies have shown that cellular senescence can also be triggered by either p21 induction or E2F1 inhibition in cells carrying mutant p53 (4, 19, 20, 43). In contrast, we show here that p21 overexpression downregulates E2F1 and CIP2A expression in p53-mutant MDA-MB-231 cells, in which CIP2A depletion provokes senescence induction (Figs. 2F and 3C). To study whether CIP2A downregulation is required for senescence induced by p21, CIP2A adenovirus-infected MDA-MB-231 cells were reinfected with either control or p21-expressing adenovirus. As shown in Fig. 4J and K, stable expression of CIP2A rescued the senescence phenotype induced by p21 overexpression. Moreover, inhibition of Rb had no effect on CIP2A depletion–induced senescence in MCF-7 cells (Supplementary Fig. S3B and S3C), further indicating that CIP2A regulates senescence downstream of the p53–p21–Rb pathway.

Taken together, these results reveal the E2F1-CIP2A positive feedback loop and its role in determining cellular senescence induction in breast cancer cell lines. Interestingly, our results suggest that even transient stabilization of E2F1 upon p53 reactivation is sufficient to prevent initiation of senescence. Importantly, the functional role of this newly identified feedback loop is not restricted to p53-induced senescence, but contributes also to senescence induction by p21 in p53-mutant cells.

**CIP2A Inactivation Induces Senescence and Growth Arrest and Restricts Tumorigenesis in a Breast Cancer Mouse Model**

We have recently generated a CIP2A hypomorphic mouse model (CIP2AHOZ) using gene trap technology (44). Despite efficient inhibition of CIP2A expression in all examined tissues, CIP2AHOZ mice do not show obvious developmental or growth defects (Supplementary Fig. S4A–S4G; ref. 44). However, consistent with the senescence phenotype observed in CIP2A-depleted cancer cells (Fig. 3A–C), MEFs isolated from CIP2AHOZ mouse embryos (Fig. 5A) underwent growth arrest after only a few passages (Fig. 5B), and displayed increased SA-β-gal staining and flattened cell morphology (Fig. 5C and D). Importantly, Nutlin-3 treatment of WT MEFs induced a level of senescence equal to that observed in CIP2AHOZ cells spontaneously, but Nutlin-3 could not further increase senescence in CIP2AHOZ cells (Fig. 5D). Moreover, overexpression of CIP2A also rescued Nutlin-3–induced downregulation of E2F1 also in MEFs, indicating that CIP2A-mediated E2F1
stabilization is a conserved mechanism between humans and rodents (Fig. 5E).

To study whether, in addition to p53 activation (10), the loss of CIP2A also suppresses tumorigenesis, we analyzed mammary tumor initiation and progression in the MMTV<sup>neu</sup> breast cancer mouse model crossed with CIP2A<sup>HOZ</sup> mice. Notably, 35% of MMTV<sup>neu</sup> tumors are known to harbor mutations in the p53 DNA binding domain, a frequency relatively similar to that seen in unselected human breast cancer material (45). In accordance with results from human samples (22, 23), normal mouse mammary glands expressed very low levels of CIP2A (Fig. 6A). However, CIP2A mRNA expression was greatly increased in MMTV<sup>neu</sup> × CIP2A<sup>HOZ</sup> (neu/WT) tumors (P = 0.003; Fig. 6A), and efficient inhibition of CIP2A expression in MMTV<sup>neu</sup> × CIP2A<sup>AA/∆</sup> (neu/HOZ) tumors was confirmed by reverse transcriptase PCR (RT-PCR) analysis (Fig. 6A). Interestingly, as compared with neu/WT mice, neu/HOZ mice had fewer Ki-67–positive epithelial cells in macroscopically tumor-free mammary glands (Fig. 6B and C and Supplementary Fig. S4H). In line with these observations, the average number of mammary tumors per mouse was significantly reduced in neu/HOZ mice (P = 0.0220; Fig. 6D). Furthermore, follow-up of the tumors that developed in mice with either of the genotypes showed that the time for tumor growth, from the day of tumor appearance to the day when the mice had to be sacrificed because the 20-mm maximum size of the largest tumor allowed was reached, was significantly delayed in neu/HOZ mice (P = 0.0030; Fig. 6F).

In concert with the in vitro results shown above, mammary tumors in CIP2A-deficient mice displayed gene expression changes indicative of senescence induction (Fig. 6F).
these senescence-inhibiting genes downregulated in new/HOZ tumors (6, 7, 46–48). Twist1 and Id1 are particularly interesting, as they have both recently been shown to block oncogene-driven senescence in breast cancer cells (46, 48). Importantly, expression of the p53-induced senescence marker DcR2 (11) was also increased in CIP2A-deficient new/HOZ tumors at the protein level (Fig. 6H). Moreover, we observed spontaneous induction of SA-β-gal expression in cultured cells isolated from new/HOZ tumors (Fig. 6G and H). Together, these results validate the senescence phenotype of CIP2A-deficient breast cancer cells in vivo.

To confirm the in vivo role for CIP2A in the inhibition of senescence in another setting, and without potentially confounding effects of mouse strain crossings, the effect of CIP2A expression in dimethylbenzanthracene (DMBA) treatment-induced senescence in mouse skin (5) was examined. As hypothesized, we detected significantly more SA-β-gal staining in DMBA-treated CIP2AHOZ mouse skin as compared with WT mouse skin (Supplementary Fig. S4I and S4J). Together, these results validate induction of senescence as a plausible cause for decreased mammary tumorigenesis in CIP2A-deficient mice.

To examine whether the above-described role for CIP2A in promoting E2F1 expression would also be observed in an in vivo setting, we conducted Western blot analysis of tumor lysates. Indeed, E2F1 expression was decreased in new/HOZ tumors as compared with new/WT tumors (Fig. 6I and J). In addition, mRNA expression of direct E2F1 target genes, Rbl1 and Id1, was decreased in new/HOZ tumors (Supplementary Fig. S4K).

Taken together, these results provide the first genetic evidence for the requirement of CIP2A for tumor formation and growth. Moreover, these findings validate CIP2A’s functional role as an in vivo inhibitor of senescence induction in breast cancer (Fig. 6B–H).

CIP2A Confers Resistance of Human Breast Tumors to Senescence-Inducing Chemotherapy

Our results thus far have shown that CIP2A expression determines cellular senescence induction in response to p53 and p21 activation. To study the clinical relevance of these findings, the expression levels of, and the prognostic role for, CIP2A were studied in a cohort of breast cancer tumor samples from patients with advanced disease (n = 1,010; ref. 49). Interestingly, CIP2A was overexpressed in 79% of the breast cancers in this population of women (Fig. 7A), of whom 89% had axillary node–positive breast cancer and the rest had high-risk node-negative cancer (49). This frequency is far greater than the frequency of CIP2A overexpression in unselected human breast cancers (approximately 40%; Fig. 1B; ref. 22). Also in this cohort, CIP2A expression is significantly associated with high p53 immunopositivity (Fig. 7A) and with several features linked with aggressive disease (Fig. 7A). The difference in overall survival of patients with low or high CIP2A expression did not quite reach statistical significance in the entire patient population (P = 0.073; Supplementary Fig. S5A). However, in HER2-negative breast cancers, representing the great majority (77%) of the studied patient material (45), high tumor CIP2A expression was significantly associated with poor overall survival (P = 0.011; Fig. 7B) and distant recurrence or death (P = 0.024; Supplementary Fig. S5B). In multivariate analysis, assessing the independent role for CIP2A as a prognostic factor in HER2-negative breast cancers, tumor CIP2A expression tended to be associated with poor outcome [P = 0.058; for CIP2A− vs. CIP2A+, HR = 4.26; 95% confidence interval (CI), 1.29–14.08; P = 0.017; for CIP2A− vs. CIP2A+, HR = 1.54; 95% CI, 0.75–3.15; P = 0.241], whereas tumor size (≥2.0 cm vs. ≤2.0 cm), axillary nodal status (positive vs. negative), histologic grade (poorly vs. moderately vs. well differentiated), and p53 expression (positive vs. negative) were not associated with survival (P ≥ 0.10 for each). However, absent estrogen receptor expression was independently associated with poor survival in HER2-negative breast cancer (HR = 2.18; 95% CI, 1.12–4.23; P = 0.022). We speculate that CIP2A does not have prognostic value in HER2-positive cancers (P = 0.687; Supplementary Fig. S5C), even though it supports mammary tumorigenesis in the HER2-driven mouse model (Fig. 6E), because human cancers have a more complex pattern of oncogenically active proteins, the combined activity of which masks CIP2A’s prognostic effect.

To study the role of tumor CIP2A in the response of HER2-negative cancers to adjuvant therapy, the association of CIP2A expression with survival of patients was studied in patient groups stratified by the type of chemotherapy administered (Fig. 7C). In these groups, patients were randomly assigned to receive either single-agent docetaxel or vinorelbine (3 cycles) followed (in both groups) by 3 cycles of fluorouracil, epirubicin, and cyclophosphamide (FEC; ref. 49). Notably, CIP2A overexpression significantly correlated with poor overall survival in the subgroup of patients who were assigned to receive vinorelbine followed by FEC (P = 0.019; Fig. 7D), whereas CIP2A expression was not significantly associated with survival of patients assigned to docetaxel followed by FEC (P = 0.373; Supplementary Fig. S5D).

Vinorelbine is a semisynthetic vinca alkaloid used to treat several kinds of human cancer, including non–small cell lung cancer and advanced breast cancer (50, 51). Interestingly, another vinca alkaloid, vincristine, has been shown to induce senescence in MCF-7 cells (52). On the basis of this information, and the novel role for the E2F1-CIP2A feedback loop in preventing chemotherapy-induced E2F1 senescence, we hypothesized that the favorable survival of the patients with CIP2A-negative cancer in the vinorelbine group could be linked with sensitivity of these cancers to vinorelbine-induced inhibition of E2F1. Indeed, vinorelbine-treated MCF-7 cells mimicked the E2F1 and CIP2A inhibition-associated phenotype by displaying increased SA-β-gal positivity and flattened cellular morphology (Fig. 7E). Importantly, induction of a senescence phenotype by vinorelbine was preceded by inhibition of both E2F1 and CIP2A protein expression at the 24-hour time point (Fig. 7F). Interestingly, vinorelbine-induced E2F1 downregulation was not accompanied by either p53 or p21 induction (Fig. 7G and Supplementary Fig. S5E and S5F), but similarly to Nutlin-3 treatment, it was associated with inhibition of E2F1 mRNA expression (Fig. 7G). To study whether CIP2A-deficient breast cancer cells are indeed more sensitive to vinorelbine-elicited E2F1 inhibition, MCF-7 cells transfected with either scrambled or CIP2A siRNA were treated with vinorelbine for 12 hours, at which time point, vinorelbine did not yet inhibit CIP2A expression in parental cells (Fig. 7H).
Figure 7. E2F1-CIP2A Feedback Loop Defines Senescence Sensitivity

A. CIP2A confers resistance of human breast tumors to senescence-inducing chemotherapy. A, CIP2A expression in human breast cancer tumors in FinHer study. CIP2A is expressed in 79% of breast tumors and correlates with high p53 immunopositivity and with other poor prognostic factors. P values by χ² test, except for Ki-67 and tumor diameter the Kruskal–Wallis test was used. B, CIP2A expression significantly correlates with survival of patients with HER2-negative tumors. CIP2A+, CIP2A-negative tumor; CIP2A*; moderately CIP2A-positive tumor; CIP2A++, high CIP2A-expressing tumor. P = 0.011 by log-rank test. C, stratification scheme of patients with HER2-negative tumors to receive therapies including either vinorelbine followed by FEC (n = 340) or docetaxel followed by FEC (n = 343). D, CIP2A overexpression is significantly associated with poor survival of vinorelbine + FEC-treated HER2-negative patients. P = 0.019 by log-rank test. E, 5A-β-gal staining of MCF-7 cells treated with vinorelbine (VRB; 30 nmol/L) for 5 days. F, Western blot analysis of E2F1 and CIP2A expression in MCF-7 cells treated with VRB (20 and 30 nmol/L) for 24 hours. G, RT-PCR analysis of p53, p21, E2F1, and CIP2A mRNA expression in MCF-7 cells treated with VRB (20 and 30 nmol/L). Shown is mean ± SEM of 2 independent experiments. H, Western blot analysis of E2F1 and CIP2A expression in either scrambled (scr) or CIP2A siRNA-transfected MCF-7 cells treated with VRB (20 and 30 nmol/L) for 12 hours. Quantitation of E2F1 expression normalized to β-actin expression is shown below the graph. I, Western blot analysis of E2F1 in either control (AdCTL) or CIP2A (AdCIP2A) adenovirus-transduced (MOI = 40) MCF-7 cells treated with VRB (10 and 30 nmol/L) for 12 hours. Quantitation of E2F1 expression normalized to β-actin expression is shown below the graph. J, schematic of the positive feedback loop between E2F1 and CIP2A in regulation of cellular senescence sensitivity downstream of p53. Inactive molecules and functions are shown in gray. In nontransformed cells (left), either oncogene- or chemotherapy-induced p53 activity inhibits E2F1 expression, resulting in subsequent inhibition of CIP2A expression. CIP2A inhibition further inhibits E2F1 protein expression by a posttranslational mechanism involving PP2A. Loss of E2F1-CIP2A positive feedback activity provokes cellular senescence and hence tumor suppression. In tumorigenic cells (right), in which p53 activity is inhibited either by mutations or by enhanced proteolytic degradation, the E2F1-CIP2A positive feedback loop is active, resulting in inhibition of senescence induction and hence tumor progression. Importantly, in addition to p53 inactivation, activity of E2F1-CIP2A feedback may be stimulated by ETS1 and MYC, which enhance CIP2A expression.
As expected, CIP2A siRNA inhibited E2F1 protein expression in nontreated cells, and, importantly, CIP2A deficiency dramatically potentiated E2F1 downregulation in vinorelbine-treated cells (Fig. 7H). Furthermore, exogenous CIP2A expression totally prevented E2F1 downregulation in vinorelbine-treated MCF-7 cells (Fig. 7I).

These results show clinical relevance for CIP2A in the progression and chemotherapy response of human breast cancers. Importantly, these findings imply that CIP2A could be a useful predictive marker for selecting patients with HER2-negative breast cancer, which currently lacks efficient targeted therapy options, for vinca alkaloid-containing chemotherapies. Moreover, these results indicate that the E2F1–CIP2A feedback mechanism is involved in chemotherapy resistance toward compounds that inhibit E2F1 expression independently of p53 or p21 activation.

**DISCUSSION**

Mounting evidence indicates that the tumor suppression function of p53 relies on its capacity to induce senescence (1, 8–10, 53). In this study, we identify inhibition of CIP2A expression as a previously unrecognized mechanism required for senescence induction by activated p53 and p21 (Fig. 7J). CIP2A’s role as a functional p53 target is supported strongly by both unbiased bioinformatics analysis of the transcriptome in CIP2A-depleted cells (Fig. 1K) and by senescence experiments (Figs. 3A, C, E, and 1C and D). Importantly, CIP2A is positively regulated by p53 inactivation regardless of whether p53 activity is inhibited by Mdm2 (Fig. 1D and E), by mutations (Fig. 1H), or by RNAi (Fig. 1C). In addition to in vitro conditions, CIP2A expression correlates with p53 mutation in human breast cancer (Figs. 1A and B and 7A), and in vivo reactivation of p53 in transgenic lymphomas expressing p53ER fusion protein potently inhibits CIP2A protein expression (Fig. 1I and J). Furthermore, we show that loss of CIP2A restricts mammary carcinogenesis in a mouse model known to harbor p53 mutations (Fig. 6E; ref. 45). Moreover, a recent study showed that in vitro gastric cancer, CIP2A has the most significant prognostic role in p53-immunopositive tumors (24). These findings together validate the in vivo relevance of CIP2A as a novel p53 target protein. Of note, CIP2A is not a direct p53 target gene, but is regulated via the p21–E2F1 axis (Fig. 2), albeit its expression is not sensitive to cell-cycle inhibition (24). Moreover, we show that CIP2A inhibition is required for p21-induced senescence in p53-mutated cancer cells (Fig. 4F and K). These results provide a novel mechanistic explanation for the recently shown in vivo function of p21 in inducing senescence and delaying tumor onset (4, 54). The results of this study strongly indicate that inhibition of CIP2A oncoprotein expression is a novel tumor suppression mechanism driven by the p53–p21 pathway (Fig. 7J). Moreover, these results explain how inactivation of the p53–p21 pathway promotes senescence resistance in cancer.

Inhibition of E2F transcriptional activity provokes senescence in human tumor cells and inhibits tumor growth (19–21). Nevertheless, E2F1 target genes involved in preventing senescence induction in cancer cells have been elusive. Our results show that activation of the p53–p21 pathway by Nutlin-3 simultaneously induces dephosphorylation of Rb, and transcriptional inhibition of E2F1 gene expression (Fig. 4A and B). We postulate that transcriptional inhibition of E2F1 by both Nutlin-3 and vinorelbine (Figs. 4B and 7C) explains consequent inhibition of CIP2A expression and triggers inhibition of a positive feedback loop between E2F1 and CIP2A (Fig. 7J). Our data indicate that CIP2A supports E2F1 protein expression at the posttranslational level in both human and mouse cells. In addition to overexpression data, we also confirmed that CIP2A depletion caused inhibition of E2F1 protein expression (Fig. 7H). In search of a mechanistic explanation for CIP2A-mediated stabilization of E2F1 protein expression, we observed that CIP2A promotes E2F1 serine 364 phosphorylation, and this phosphorylation has been previously shown in another context to be associated with increased stability of E2F1 (17, 41). Moreover, we observed that inhibition of a regulatory subunit of PP2A, B55α, increases E2F1 serine 364 phosphorylation and reverses Nutlin-3–induced downregulation of E2F1 (Fig. 4F and G). Previously, we showed that inhibition of the P PP2R2A gene, encoding B55α, reverses CIP2A depletion–induced antiproliferative and gene expression effects (34). Interestingly, deletion of the B55α gene was recently identified as a potential driver mutation specifically in the luminal B type of breast cancer (55). These results indicate that the B55α-containing PP2A tumor suppressor complex needs to be inhibited during breast cancer progression either by genetic mutations or via overexpression of CIP2A. Importantly, our data also indicate that mechanisms other than p53 inactivation–induced E2F1 expression may drive high CIP2A expression in human breast cancer (Figs. 1B and 7A). We postulate that in these cases ETS-1– and MYC-mediated CIP2A expression (24, 30) supports E2F1 expression and thereby confers on these cells resistance to senescence induction (see Fig. 7 for schematic presentation).

Although CIP2A expression has been shown to predict for poor patient survival in many different human cancer types (refs. 24, 28; Supplementary Table S1), such evidence has thus far been lacking for breast cancer. In this study, we show that CIP2A has a prognostic role in HER2-negative breast cancer, for which novel therapy targets are in high demand. Interestingly, low E2F1 mRNA expression levels were found specifically in HER2-negative breast tumors (56). Therefore, it can be envisioned that the prognostic value of CIP2A becomes more apparent in HER2-negative cancers in which CIP2A-mediated posttranslational increase of E2F1 protein becomes critical for tumor progression. Moreover, the observation that the E2F1 response to senescence-inducing vinorelbine chemotherapy is dependent on CIP2A status provides a plausible mechanistic explanation for the favorable survival of patients who have CIP2A/HER2-negative breast cancer and who were treated with vinorelbine before FEC (Fig. 7D).

Prosensence therapies are emerging as an alternative approach for cancer treatment (6, 7). However, the majority of the strategies suggested thus far for therapeutic senescence induction rely on activation of p53 and other cellular checkpoint mechanisms (6, 7). Although hypothetically reasonable, these strategies suffer from serious shortcomings because in the majority of human cancers several checkpoint mechanisms are functionally impaired. Therefore, identification of the E2F1–CIP2A–positive feedback mechanism provides a plausible mechanistic explanation for the favorable survival of patients who have CIP2A/HER2-negative breast cancer and who were treated with vinorelbine before FEC (Fig. 7D).
loop as a novel prosenecence therapeutic target mechanism that functions downstream of inactivated p53, in which inhibition induces senescence independently of p53 activation, is a fundamentally important finding. As an example of the in vivo importance of the p53-independent senescence-inducing mechanisms, Lin and colleagues (4) recently showed a role for p21-induced senescence in tumor suppression. In that regard, our data indicate that CIP2A expression not only inhibits p53-induced senescence (Fig. 3E, F, I, and J) but also p21-induced senescence in p53-mutant breast cancer cells (Fig. 4f and K). As p53 inhibition promotes CIP2A expression (Figs. 1 and 2), these results together indicate that senescence resistance in p53-mutant tumors is caused by a combined effect of impaired p53 checkpoint activity and increased activity of the E2F1-CIP2A feedback loop. Therefore, CIP2A deregulation could be considered a novel gain-of-function for mutant p53 in cancer (13). Importantly, the feasibility of targeting the identified E2F1-CIP2A positive feedback loop for prosenecence therapy is supported by the lack of any obvious developmental defects in the CIP2A knockdown mouse used in this study (Supplementary Fig. S4; ref. 44). Moreover, as CIP2A is overexpressed at an exceptionally high frequency in 65% to 90% of tumor samples of most major human cancer types (Supplementary Table S1), its inhibition could serve as a general strategy to sensitize cancer cells to prosenecence therapies. These conclusions are supported by a previously reported increase in SA-β-gal activity in a CIP2A-depleted gastric cancer cell line (57).

In summary, this study identifies a hitherto unrecognized oncogenic mechanism downstream of the inactivated p53-p21 pathway. Our results show that although E2F1 stimulates CIP2A expression in cells with an inactive p53-p21 pathway, inhibition of the E2F1-CIP2A feedback loop is essential for senescence induction (Fig. 7). Moreover, as inhibition of the E2F1-CIP2A feedback loop also induces senescence in p53-mutant cells, and pRb is not needed for CIP2A inhibition-induced senescence (Supplementary Fig. S3), these results indicate that inhibition of E2F1 and CIP2A can induce senescence in cancer cells without activation of the upstream p53-p21 pathway. In general, these findings suggest that senescence induction in cancer cells is determined by the activity of this newly identified feedback mechanism between E2F1 and CIP2A, rather than simply by the strength of the senescence-inducing stimuli (Fig. 7). Finally, results of this study should encourage development of approaches both to target E2F1-CIP2A feedback mechanism and to stratify patients to senescence-inducing cancer therapies based on tumor CIP2A status.

METHODS

Cell Culture and Drug Treatments

MCF-7, MDA-MB-231, HeLa, and SAOS-2 cell lines were obtained from American Type Culture Collection. HCT116 and its clonal p53 mutant breast cancer cells (Fig. 4J and K). As p53 inhibition promotes CIP2A expression (Figs. 1 and 2), these results together indicate that senescence resistance in p53-mutant tumors is caused by a combined effect of impaired p53 checkpoint activity and increased activity of the E2F1-CIP2A feedback loop. Therefore, CIP2A deregulation could be considered a novel gain-of-function for mutant p53 in cancer (13). Importantly, the feasibility of targeting the identified E2F1-CIP2A positive feedback loop for prosenecence therapy is supported by the lack of any obvious developmental defects in the CIP2A knockdown mouse used in this study (Supplementary Fig. S4; ref. 44). Moreover, as CIP2A is overexpressed at an exceptionally high frequency in 65% to 90% of tumor samples of most major human cancer types (Supplementary Table S1), its inhibition could serve as a general strategy to sensitize cancer cells to prosenecence therapies. These conclusions are supported by a previously reported increase in SA-β-gal activity in a CIP2A-depleted gastric cancer cell line (57).

In summary, this study identifies a hitherto unrecognized oncogenic mechanism downstream of the inactivated p53-p21 pathway. Our results show that although E2F1 stimulates CIP2A expression in cells with an inactive p53-p21 pathway, inhibition of the E2F1-CIP2A feedback loop is essential for senescence induction (Fig. 7). Moreover, as inhibition of the E2F1-CIP2A feedback loop also induces senescence in p53-mutant cells, and pRb is not needed for CIP2A inhibition-induced senescence (Supplementary Fig. S3), these results indicate that inhibition of E2F1 and CIP2A can induce senescence in cancer cells without activation of the upstream p53-p21 pathway. In general, these findings suggest that senescence induction in cancer cells is determined by the activity of this newly identified feedback mechanism between E2F1 and CIP2A, rather than simply by the strength of the senescence-inducing stimuli (Fig. 7). Finally, results of this study should encourage development of approaches both to target E2F1-CIP2A feedback mechanism and to stratify patients to senescence-inducing cancer therapies based on tumor CIP2A status.

Antibodies

For immunoblotting, the following antibodies were used: CIP2A: rabbit polyclonal (57) and mouse monoclonal 2G10-3B5 (Santa Cruz); p21: rabbit polyclonal C-19 (Santa Cruz); p53: mouse monoclonal DO-1 (Santa Cruz) and rabbit polyclonal CMS (Vector Laboratories); β-actin: mouse monoclonal (Sigma); Rb: rabbit polyclonal C-15 (Santa Cruz); B55: mouse monoclonal 2G9 (Cell Signaling); Ser 807/811 phosphorylated Rb: rabbit polyclonal (Santa Cruz); E2F1 K9H5: mouse monoclonal (Santa Cruz); serine 364 phosphorylated E2F1: rabbit polyclonal (Abcam); and DcR2: rabbit polyclonal (Abcam).

Immunohistochemical and Statistical Analysis of Human Breast Cancer Patient Samples

CIP2A immunostaining in both FinProg and FinHer breast cancer patient cohorts was conducted with polyclonal rabbit antibody (58). CIP2A was immunostained and analyzed from both cohorts of human breast cancer patient tumor samples (FinProg and FinHer studies), as described previously (34). In the FinProg cohort of patients with breast cancer, p53 and Ki-67 immunostaining of breast tumor samples and analysis of tumor size and tumor grades were conducted as previously described (59). In the FinHer cohort of patients with breast cancer, HER2 and Ki-67 immunostaining; analysis of tumor diameter, tumor size, and tumor grade; and statistical analysis of total and cumulative survival and percentage of alive patients in different subgroups were conducted similarly as before (49). The p53 immunostaining from the FinHer cohort was done following same protocol as published for the FinProg study (59). An ethics committee at Helsinki University Hospital (Helsinki, Finland) approved the FinHer study (HUCH 426/E6/00). Regarding FinProg material, permission to use formalin-fixed, paraffin-embedded tissues for research purposes was provided by the Ministry of Social Affairs and Health, Finland (permission 123/08/97).

Animal Experiments

MMTV<sup>neu</sup> mice (60) expressing oncogenic HER2 under the control of the mouse mammary tumor virus promoter specifically in the mouse mammary gland were purchased from The Jackson Laboratory and crossed with CIP2A heterozygous genetrap hypomorphic mutant mice (CIP2A<sup>−/−</sup>, ref. 44). MMTV<sup>neu</sup>/CIP2A<sup>−/−</sup> mice were intercrossed to produce MMTV<sup>neu</sup>/CIP2A<sup>−/−</sup> <sup>−/−</sup> mice and MMTV<sup>neu</sup>/CIP2A<sup>−/−</sup> mice. Mice were genotyped by PCR analysis of genomic DNA for MMTV<sup>neu</sup> transgene according to The Jackson Laboratory’s protocol and for CIP2A genetrap, as previously described (44). CIP2A genotyping results were confirmed with mRNA analysis by RT-PCR. Mice were checked for tumor appearance twice a week. Formed tumors were palpated twice a week, and mice were sacrificed when tumor diameter reached 20 mm. Tumor size was measured by palpation and by weighing after preparation of the tumor from sacrificed mice. Immunohistochemical staining for Ki-67 and DcR2 and hematoxylin and eosin (H&E) staining were conducted as previously described (41). Tumor cells were isolated by forcing cells through a 70-μm pore filter (BD Biosciences). Cells were cultured with Dulbecco modified Eagle medium (DMEM)/F12 Ham medium containing 10% serum, insulin, hydrocortisone, and mouse EGF. MEFs were isolated from WT and CIP2A<sup>−/−</sup> embryos at 13.5 days of gestation, and cultured in DMEM containing 15% serum.

In DMBA treatment, the dorsal skin of WT and CIP2A<sup>−/−</sup> mice was treated with DMBA (20 μg in 200 μL of acetone) 3 times a week for 2 weeks. A day before the first treatment, mouse backs were shaved, and mice were sacrificed 24 hours after the last treatment. Lymphoma lysates from EµMyc-p53ER mice systemically treated with either tamoxifen or peanut oil were prepared as described previously (33). All animal work protocols were approved by the Regional State Administrative Agency for Southern Finland (ESLH-2007-08517, ESLH-2009-00515/Ym-23).
Proliferation Assay and SA-β-gal Staining

Proliferation capacity of MEFs was studied by calculating cell numbers of MEFs from 3 different WT and CIP2A−/− embryos seeded to 14,000 cells/cm² and divided when 70% to 80% confluent. Cells were cultured for 46 days. To detect senescent cells, cells and mouse skin sections were fixed and stained for SA-β-gal at pH 6.0 (Sigma) according to the manufacturer’s protocol. Senescent cells in n vitro assays were quantified under the microscope by counting morphologically flattened and SA-β-gal-positive cells. SA-β-gal staining in mouse skin was quantified by counting positively stained areas from 2 to 3 sections per mouse.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Senescence Sensitivity of Breast Cancer Cells Is Defined by Positive Feedback Loop between CIP2A and E2F1

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