Paracrine Apoptotic Effect of p53 Mediated by Tumor Suppressor Par-4

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SUMMARY

The guardian of the genome, p53, is often mutated in cancer and may contribute to therapeutic resistance. Given that p53 is intact and functional in normal tissues, we harnessed its potential to inhibit the growth of p53-deficient cancer cells. Specific activation of p53 in normal fibroblasts selectively induced apoptosis in p53-deficient cancer cells. This paracrine effect was mediated by p53-dependent secretory activation of the tumor suppressor Par-4. Accordingly, the activation of p53 in normal mice, but not p53−/− or Par-4−/− mice, caused systemic elevation of Par-4, which induced apoptosis of p53-deficient tumor cells. Mechanistically, p53 induced Par-4 secretion by suppressing the expression of its binding partner, UACA, which sequesters Par-4. Thus, normal cells can be empowered by p53 activation to induce Par-4 secretion for the inhibition of therapy-resistant tumors.

INTRODUCTION

Lung cancer is the leading cause of cancer deaths in both men and women in the United States (Siegel et al., 2012). Activation of the Ras oncogene and loss of tumor suppressor p53 are the two most commonly occurring alterations in lung cancer. Given that p53 function is essential for growth arrest and cell death by diverse chemotherapeutic agents and ionizing radiation, loss of p53 function in the tumors may confer therapeutic resistance ultimately leading to death of the patients (Levine, 1997; Chen et al., 2010). P53 knockout or p53 mutant mice develop spontaneous as well as inducible tumors (Donehower et al., 1992; Jacks et al., 1994), whereas p53 transgenic mice exhibit tumor-free survival with an increased life span (García-Cao et al., 2002). Because up- or downregulation of transcriptional target genes involved in cell-cycle progression or apoptosis plays an important role in the tumor suppressor function of p53, it is not surpris-
embryonic fibroblasts (MEFs) from p53 +/+ or p53 /C0 /C0 mice with p53-null H1299 and PC-3 cells and p53 mutant HOP92 cells. The cell cultures were treated with Nutlin-3a, a specific activator of p53 (Vassilev et al., 2004). Nutlin-3a induced apoptosis in p53-deficient cancer cells that were cocultured with p53 +/+ MEFs but not with p53 /C0 /C0 MEFs (Figure 1A, left). As expected, the p53-deficient cancer cells and the MEFs were resistant to apoptosis by Nutlin-3a when cultured individually (Figure 1A, right). Because p53 may function by partial inhibition of NF-κB activity (Dey et al., 2007), we combined Nutlin-3a with PS-1145, a small molecule that specifically inhibits IKK (see Burikhanov et al., 2013). By itself, PS-1145 does not induce apoptosis of normal or lung cancer cells (Figure 1A, right; Burikhanov et al., 2013). However, treatment of the cocultures with PS-1145 induced apoptosis in cancer cells, and the combination of Nutlin-3a plus PS-1145 highly augmented that effect (Figure 1A).

To determine whether extracellular factors secreted by the MEFs in response to Nutlin-3a and/or PS-1145 treatment contributed to apoptosis of the cancer cells, the MEFs were treated with these small molecules, and conditioned medium (CM) was transferred to p53-deficient lung cancer cells or normal lung cells. The CM from p53 +/+ MEFs but not the CM from p53 /C0 /C0 MEFs treated with Nutlin-3a or PS-1145 induced apoptosis of H1299 cells (Figure 1B). Apoptosis of H1299 cells was scored after 24 hr by ICC for active caspase-3.

Figure 1. P53 Activation in Normal Cells Produces Paracrine Apoptosis in p53-Deficient Cancer Cells

(A) Activation of p53 in MEFs induces apoptosis of cocultured p53-deficient cancer cells. Cocultures of p53 +/+ or p53 /C0 /C0 MEFs with the indicated cancer cells were treated with vehicle (v), Nutlin-3a (N, 10 μM), and/or PS-1145 (P, 10 μM) for 24 hr. The cells were subjected to ICC for cytokeratins to detect epithelial cancer cells and for active caspase-3 to determine apoptotic cells.

(B) Apoptosis of cancer cells by CM from p53-activated MEFs. PS-1145 /C0 /C0 MEFs were treated with vehicle (v), Nutlin-3a (N, 10 μM), and/or PS-1145 (P, 10 μM) for 24 hr, and their CM was transferred to normal (HEL) or p53-deficient cancer (H1299) cells. Apoptotic cells were scored after 24 hr by ICC for active caspase-3.

(C) Coparallel activation of p53 and inhibition of NF-κB activity additively increases Par-4 secretion in fibroblasts. MEFs were treated with vehicle (v), Nutlin-3a (N), and/or PS-1145 (P) for 24 hr, and their CM, as well as whole-cell lysates, was subjected to western blot analysis for Par-4. β-actin was used to normalize loading of lysate. Coomassie blue-stained albumin in the serum was used to normalize loading of CM. Par-4 secretion, but not Col1A1 secretion, was dependent on the p53 status of the cells. Fold change in secreted Par-4 is shown.

(D) Par-4 secretion is critical for the paracrine effect resulting from simultaneous p53-activation and NF-κB-inhibition. The CM from p53 +/+ cells treated with Nutlin-3a + PS-1145 was incubated with polyclonal antibody for Par-4 or PTEN control and then added to H1299 cells. Apoptosis of H1299 cells was scored after 24 hr by ICC for active caspase 3 (left panel). Par-4+/+ and Par-4 /C0 /C0 MEFs were treated with Nutlin-3a + PS-1145 (N+P) or vehicle, and then the CM was applied to the indicated cell lines, and apoptotic cells were scored by ICC for active caspase-3 (middle panel). Expression of Par-4 in CM was verified on western blots (right panel).

Asterisks in (A), (B), and (D) indicate statistical significance (p < 0.001) by the Student’s t test; **N+P is significantly (p < 0.001) more effective than individual treatments based on two-way ANOVA. Error bars indicate SD.
We then examined the CM for secreted proteins, especially TRAIL, maspin, IGFBP3, and Par-4, which are known to act extracellularly and induce cancer-specific apoptosis. The CM from p53+/+ MEFs treated with Nutlin-3a or PS-1145 showed elevated levels of Par-4 protein, and combination of Nutlin-3a plus PS-1145 additively increased the secretion of Par-4 (Figure 1C). None of the other proteins showed elevated secretion with Nutlin-3a plus PS-1145 (R.B. and T.S.-B., unpublished data). By contrast, p53−/− MEFs accumulated Par-4 protein in the lysate but failed to secrete it in response to these treatments (Figure 1C). Moreover, doxorubicin, which is known to activate p53, induced Par-4 secretion from p53+/+ but not p53−/− MEFs, and the CM collected from p53+/+ but not p53−/− MEFs induced apoptosis of p53-deficient cancer cells (Figure S1D). Together, these findings suggest that p53 regulates the secretion of Par-4 protein, which selectively induces apoptosis in cancer cells.

**P53 Induces Systemic Expression of Par-4 in Normal Mice**

We interrogated whether p53 regulates the secretion of Par-4 in vivo. The serum of untreated C57BL/6 p53+/+ mice contained higher levels of Par-4 relative to p53−/− mice (Figure 2A, left). When p53+/+, p53−/−, and p53+/− mice were injected with Nutlin-3a plus PS-1145 or vehicle control, and serum samples were collected for analysis of Par-4, we noted that, relative to vehicle control, Nutlin-3a plus PS-1145 (N+P) caused a ~5-fold increase in serum levels of Par-4 protein in p53+/+ mice (Figure 2A, right). By contrast, Nutlin-3a plus PS-1145 failed to elevate systemic levels of Par-4 in p53−/− mice or Par-4−/− mice (Figure 2A, right), implying that p53 function was essential from Par-4+/+ MEFs but not Par-4−/− MEFs treated with Nutlin-3a plus PS-1145 induced apoptosis of p53-deficient cancer cells (Figure 1D, middle and right).

Figure 2. Activation of p53 in Mice Induces Systemic Expression of Par-4 Proapoptotic Activity

(A) P53 function is essential for induction of systemic Par-4 in mice. Serum samples from p53+/+ mice (4 μl per lane) and p53−/− mice (8 μl per lane) (left panel) and also from p53+/+, p53−/−, and Par-4−/− mice (right panel) injected i.p. with either vehicle (v) or with Nutlin-3a + PS-1145 (N+P) were examined for Par-4 expression by western blot analysis using serum albumin to normalize loading. Data are representative of four mice per treatment. (B) Serum from Nutlin-3a plus PS-1145-treated p53+/+ mice induces ex vivo apoptosis of cancer cells. Serum samples collected from p53+/+, p53−/−, and Par-4−/− mice injected i.p. with either vehicle (lower panel) or with Nutlin-3a + PS-1145 (upper panel) was applied at a final concentration of 10% to the indicated normal or cancer cell lines. The cells were scored for apoptosis after 24 hr. Fetal bovine serum (FBS, 10%) was used as an additional control. (C) Secreted Par-4 is essential for the paracrine effect of p53 activation. Serum samples collected from p53+/+ mice treated with vehicle or Nutlin-3a + PS-1145 (N+P) were preincubated with Par-4 antibody, control PTEN antibody or no antibody (no antibody [Ab]), and then applied on lung cancer cells. After 24 hr of treatment, the cells were scored for apoptosis. Asterisks in (A) and (B) indicate statistical significance (p < 0.001) by the Student’s t test. Error bars indicate SD.

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for upregulation of Par-4 secretion in mice. Importantly, the serum from Nutlin-3a plus PS-1145-treated p53+/+ mice, but not p53−/− mice or Par-4−/− mice, induced ex vivo apoptosis of cancer cell cultures but not normal cell cultures (Figure 2B, top). By contrast, the serum from vehicle-treated mice failed to induce apoptosis over background levels in normal or cancer cells (Figure 2B, bottom). Moreover, preincubation of the serum samples collected from Nutlin-3a plus PS-1145-treated p53+/+ mice with the Par-4 antibody but not the PTEN control antibody significantly inhibited the ability of the serum to induce apoptosis of cancer cell cultures (Figure 2C). Altogether, these findings suggest that p53 activation in normal mice induces adequate levels of systemic Par-4 protein that is functionally effective in inducing apoptosis of cancer cells.

Figure 3. P53 Stimulates Par-4 Secretion by Suppressing the Expression of UACA

(A) P53 downregulates UACA. Whole-cell lysates from p53+/+ and p53−/− MEFs that were either untreated or treated with vehicle (v), Nutlin-3a (N, 10μM), or PS-1145 (P, 10 μM) for 24 hr (left three panels), or whole-tissue lysates of highly vascular organs obtained from p53+/+ and p53−/− mice (right three panels) were examined for UACA by western blot analysis.

(B) Restoration of p53 activity inhibits UACA expression and promotes Par-4 secretion. P53−/− MEFs were infected with GFP-tagged p53- or GFP-producing adenoviral constructs (left panel). Also, the mouse fibroblasts (101), which do not express any p53, and (101)-derived Val5 cells, which are engineered to stably overexpress wild-type p53 at 32°C or mutant p53 at 37°C, were grown at 37°C or shifted to 32°C to activate p53 (right panel). Expression of the indicated proteins in the CM or whole-cell lysate was examined by western blot analysis.

(C) UACA inhibits Par-4 secretion. UACA expression was knocked down in mouse (p53+/+ or p53−/− MEF) and human (HEL) cells with distinct siRNA pools from two different sources, Dharmacon (D) and Santa Cruz Biotechnology (SC), and the CM, as well as the whole-cell lysates, was subjected to western blot analysis. C, control (scrambled) siRNA.

(D) P53 activation and UACA inhibition promotes secretion by a BFA-sensitive pathway. (101)Val5 fibroblasts grown at 32°C were treated with BFA (1 μg/ml) or vehicle (v) for 3 hr (left panel). UACA expression was inhibited in MEFs (p53+/+) with Nutlin-3a plus PS-1145 (N+P; 10 μM each) (middle panel), or with an siRNA pool (from Dharmacon) (right panel), and then the cells were further placed in the presence of BFA or vehicle (v) for 3 hr. The CM, as well as the whole-cell lysates, was subjected to western blot analysis.

P53 Downregulates the Expression of UACA to Induce Par-4 Secretion

Because Par-4 does not contain a p53 consensus binding site in its DNA, Par-4 secretion is likely regulated by another downstream target of p53. To identify the primary gene target of p53, we used three criteria: (1) it should encode a protein that binds to Par-4, (2) it should be regulated by p53, and (3) it should be induced by NF-kB activity. We recently identified UACA as a strong binding partner of Par-4 (Burikhanov et al., 2013). UACA was coimmunoprecipitated from normal MEF and HEL fibroblast with Par-4 antibody, and Par-4 was reciprocally coimmunoprecipitated with UACA antibody (Figure S2A). A comparison of p53+/+ and p53−/− MEFs indicated that UACA was lower in p53+/+ normal cells and mouse tissues relative to p53−/− cells and mouse tissues (Figure 3A). UACA levels in p53+/+ and p53−/− MEFs correlated inversely with the levels of Par-4 secreted in the CM (Figure S2B). Nutlin-3a suppressed the expression of UACA in p53+/+ but not p53−/− MEFs.
Previous studies have indicated that Par-4 is secreted via the exosomal pathway (Wang et al., 2012). On the other hand, p53 has been shown to promote secretion of proteins via the exosomal pathway (Yu et al., 2009). To determine whether p53 activation leads to Par-4 secretion via the classical pathway, we used Brefeldin A (BFA), which blocks the ER-Golgi pathway. BFA inhibited the secretion of Par-4 by p53 restoration at 32°C in (10)1/Val5 fibroblasts (Figure 3D, left panel). As both Nutlin-3a and PS-1145 induced the secretion of Par-4 in a p53-dependent manner, we investigated whether Par-4 secretion following Nutlin-3a plus PS-1145 treatment occurred via the classical pathway. Nutlin-3a plus PS-1145-inducible secretion of Par-4 from p53+/+ MEFs was about 60% inhibited by BFA (Figure 3D, middle panel). These findings imply that p53 regulates the secretion of Par-4 largely by the classical pathway. Moreover, Par-4 secretion following UACA knockdown in p53+/+ MEFs was inhibited by BFA (Figure 3D, right). Collectively, these findings indicate that UACA inhibition by siRNA, Nutlin-3a plus PS-1145, or p53 activation induces Par-4 secretion via the classical pathway.

**UACA Is a Direct Target of p53**

A potential p53-binding site (el-Deiry et al., 1992) is present in the DNA corresponding to exon 1 within the coding region of UACA (Figure S3A). P53 suppresses the expression of UACA RNA, as judged by quantitative RT-PCR (qRT-PCR) (Figure S3B). To determine direct binding of p53 to its consensus binding site in UACA, we performed chromatin immunoprecipitation studies. HEL cells treated with Nutlin-3a, but not with vehicle, showed chromatin immunoprecipitation of endogenous p53 bound to the p53-consensus motif in UACA (Figure 4A). These findings were corroborated by chromatin immunoprecipitation studies in p53-deficient cells that were transfected with p53 expression construct (Figure S3C). Consistently, Nutlin-3a treatment, which activates endogenous p53, suppressed the expression of UACA in HEL cells (Figure 4B).

To determine whether p53 regulated UACA expression via its binding motif in an NF-κB activity-independent manner, we performed experiments in IKKβ−/− MEFs, which lack NF-κB activity (Burikhanov et al., 2013; Li et al., 1999). Nutlin-3a treatment of IKKβ−/− MEFs resulted in suppression of UACA expression (Figure 4B). Moreover, ectopic p53 suppressed the expression of UACA containing the p53-binding motif but did not suppress the expression of mutant-UACA, which contained mutations in the p53-binding sequence, in IKKβ−/− MEFs (Figure S4). Together, these findings suggest that p53 suppressed the expression of UACA via its binding motif in an NF-κB-independent manner. Thus, UACA is a relevant target of p53, and p53 may regulate Par-4 secretion by downregulation of UACA.

**DISCUSSION**

The tumor suppressor p53 is known to suppress tumor growth by intracellular activation of growth arrest and apoptotic cell death pathways. However, p53 is mutated in over 50% of cancers, and mutant forms of p53 may render cancer cells resistant to both chemotherapy and radiation therapy. Lung cancer, the leading cause of cancer-related deaths in the US, is often associated with inactivating mutations in p53. We determined...
whether wild-type p53 function in normal cells could be effect-
ively propelled to target lung cancer cells. The present study re-
vealed that p53 activation in normal cells induces paracrine a-
 apoptotic of p53-deficient lung cancer and prostate cancer cells.
This action of p53 is mediated by the tumor suppressor protein Par-4. The paracrine effect of p53 activation on Par-4 secretion was bolstered by coparallel inhibition of NF-κB activity in normal cells. We noted that UACA binds to Par-4 and prevents it from being secreted, and inhibition of UACA by p53 activation and/or inhibition of NF-κB activity, or by several different siRNAs, results in elevated secretion of Par-4. Importantly, Par-4 secreted by cells following coparallel activation of p53 and inhibition of NF-κB activity caused apoptosis in p53-deficient lung cancer cells but not normal cells. The physiological relevance of the findings was confirmed by studies indicating that normal cells in mice can be triggered to secrete proapoptotic Par-4 activity in circulation in a p53-dependent manner. The elevated levels of Par-4 in serum induced ex vivo apoptosis in tumor cells but not normal cells. Because our previous studies indicated a good correlation between inhibition of lung tumors in immuno-
competent mice and induction of ex vivo apoptosis by Par-4 in the serum of these mice (Zhao et al., 2011), the activation of p53 to trigger proapoptotic Par-4 protein secretion and elevate its systemic levels may be an effective strategy to induce apoptosis of cancer cells that metastasize through the circula-
tory route. Collectively, our findings suggest that secreted Par-4 mediates the paracrine apoptotic effects of p53, and that secretagogues of Par-4 may therefore empower normal cells to execute paracrine tumor growth inhibition.

Our findings indicate that Par-4 secretion following p53 activation occurs via the classical BFA-sensitive pathway. Secretion of Par-4 via this pathway is dependent on downmodulation of UACA, a functional target of p53. Chromatin immunoprecipitation experiments confirmed that p53 directly binds to its consensus motif in UACA. Importantly, p53 suppressed the expression of UACA in an NF-κB-independent manner. Given that UACA is a principal binding partner that sequesters Par-4 in normal cells, UACA inhibition by p53 is necessary to release Par-4 for secretion via the classical pathway. However, normal cells lacking p53 are deficient in Par-4 secretion despite UACA downregulation, implying that p53 function is critical for Par-4 secretion and is suggestive of an additional role for p53 in triggering Par-4 secretion. Several integral components of the classical ER-Golgi-transport vesicle/membrane fusion pathway are currently being investigated as targets for p53-dependent Par-4 secretion. Given that previous studies have indicated that p53 regulates protein secretion via the exosomal pathway, we tested whether Par-4 secretion is also regulated via the exosomes. Our findings indicate that, of the total Par-4 secreted by cells, <5% is secreted via the exosomal pathway, and the large majority is secreted by the classical pathway (T.S.-B., unpublished data).

In summary, our findings suggest that the tumor suppressor p53 regulates the secretion of the proapoptotic, tumor sup-
pressor Par-4 via the classical pathway by suppressing UACA, and that Par-4 executes the paracrine apoptotic effects of p53. As systemic Par-4 inhibits the growth of lung tumors (Zhao et al., 2011), Par-4 secretagogues can be exploited to activate p53 and unleash the power of normal cells in the tumor micro-
and macroenvironment to elevate systemic Par-4 and suppress tumor cell survival.

EXPERIMENTAL PROCEDURES

Cells and Chemical Reagents
Lung cancer cells H1299, HOP92, LLC1, prostate cancer cells PC-3, and primary lung fibroblast cells HEL were from ATCC. IKKα+/+ and IKKβ−/− MEFS were previously described (Burikhanov et al., 2013). Par-4+/+ and Par-4−/− MEFS were derived from wild-type and Par-4-null C57BL/6 mice, respectively (Figure S1C). KP lung tumor cells, and P53+/+ and p53−/− MEFS in the third passage were from Tyler Jacks (Massachusetts Institute of Technology). Nutlin-3a was from Cayman Chemicals.

Immortalized Balb/c fibroblasts (101), with no endogenous p53, and (10.1)Val5 cell line derived from 10/1 cells by stable transfection with the tempera-
ture-sensitive p53 allele encoding valine at 135 aa, were from Arnold Levine (Institute for Advanced Study, NJ). The p53 mutant in (10.1)Val5 cells exhibits wild-type conformation at 32°C, judged by p21/WAF1 induction, and mutant conformation at 37°C–39°C (Wu and Levine, 1994).

Animal Experiments
Whole-blood samples and various tissues were collected from mice 24 hr after injection via the intraperitoneal (i.p.) route with Nutlin-3a plus PS-1145 (10 and 5 mg/kg body weight, respectively) or corn oil (100 μl) as vehicle control. Serum was separated from the blood samples and used for testing. All animal procedures were performed with IACUC approval.

Statistical Analysis
All experiments were performed in triplicate to verify the reproducibility of the findings. Statistical analyses were carried out with Statistical Analysis System software (SAS Institute). Mean ± SD bars are shown. p values were calculated using the Student’s t test.

All other reagents and experimental procedures are presented in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.12.020.

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FIGURE S1
**SUPPLEMENTAL DATA**

**Figure S1 Related to Figure 1.** Par-4 in the CM is responsible for mediating the paracrine apoptotic effect of p53 in p53-deficient cancer cells.

(A) **Sensitivity of various cell lines to the paracrine apoptotic effect of p53 activation in normal cells.** Nutlin-3a belongs to a class of cis-imidazoline derivatives that selectively disrupt the interaction between p53 and MDM2 (Vassilev et al., 2004). The release of p53 from negative control by MDM2 results in direct activation of the p53 pathway, avoiding the nonspecific genotoxic damage inflicted by many classic cytotoxic drugs and radiation. Nutlin-3a induces growth arrest and cell death in cancer cells expressing functional p53, but is not directly effective in p53-mutant cancer cells; and it induces only reversible growth arrest in normal cells (Cheok et al., 2007; Kranz and Dobbelstein, 2006). To study the effect of p53 activation on paracrine apoptosis, we used Nutlin-3a alone or combined Nutlin-3a with PS-1145, a small molecule that specifically inhibits IKKβ (Hideshima et al., 2002). P53+/+ or p53−/− MEFs and HEL cells were treated with vehicle (v), Nutlin-3a (N, 10 µM) and/or PS-1145 (P, 10 µM) for 24 h and the CM was transferred to various normal (HEL) and p53-deficient cancer (H1299, HOP-92) cell lines. After 24 h, the cells were scored for apoptosis.

(B) **Par-4 secretion in response to p53 activation does not require apoptosis of the normal cells.** P53+/+ MEFs were treated with z-VAD-fmk (2 µM) and with Nutlin-3a (N, 10 µM) plus PS-1145 (P, 10 µM) or vehicle (v) for 24 h. The CM was examined for Par-4 levels by Western blot analysis.

(C) **Generation of Par-4/Pawr knock out mice.** Par-4 knock out (KO) mice were generated using the C57BL/6 mouse background. As Exon 2 contains the initiating ATG codon, it was flanked by loxP sites. The selection marker (Neomycin, Neo) was flanked by FRT sites and introduced into intron 2. Constitutive knock out allele was generated after in vivo Cre-mediated recombination that resulted in deletion of Exon 2 and loss of Par-4 function, owing to removal of the initiating ATG codon. Par-4−/− mice were intercrossed to generate Par-4−/− mice. Par-4 null mice grow normally and develop spontaneous tumors in diverse tissues as previously reported (Moreno-Bueno et al., 2007). MEFs isolated from the Par-4−/− mice and corresponding Par-4+/+ littermate control mice were studied for Par-4 secretion.

(D) **Doxorubicin induces paracrine apoptosis of cancer cells by Par-4 secretion.** PC-3 cells were treated with 100 nM doxorubicin (Dox) or vehicle for 24 h and scored for apoptosis by ICC for active caspase-3 (middle panel). Note 100 nM Dox does not directly induce apoptosis in PC-3 cells. Par-4+/+ or Par-4−/− MEFs were treated with 100 nM doxorubicin (Dox) or vehicle for 24 h and the CM from these cells was transferred to PC-3 cells; after 24 h the PC-3 cells were scored for apoptosis (right panel). Elevation of Par-4 in the CM of doxorubicin (Dox)- or vehicle (v)-treated Par-4+/+ or Par-4−/− MEFs was confirmed by Western blot analysis (left panel).
Figure S2 Related to Figure 3. UACA binds to Par-4 to inhibit Par-4 secretion, and p53 down-regulates UACA to restore Par-4 secretion.

(A) UACA binds to Par-4 in fibroblasts. Whole-cell lysates of human lung fibroblasts HEL and p53<sup>+/+</sup> MEFs were subjected to co-immunoprecipitation with Par-4, UACA, or p65/NF-κB (control) antibody. The immunoprecipitates and input lysates were examined by Western blot analysis. Similar to our findings in epithelial cells (Burikhanov et al., 2013), UACA binds to Par-4 in fibroblasts.

(B) UACA down-regulation by p53 correlates with Par-4 secretion. CM or whole-cell lysates from p53<sup>+/+</sup> and p53<sup>−/−</sup> MEFs were subjected to Western blot analysis for UACA or Par-4 expression. Note UACA is down-regulated in p53<sup>+/+</sup> cells. Consistently, UACA-null mice grow normally and do not exhibit significant phenotypes (Kiso et al., 2012; Sakai et al., 2010), indicating that UACA function is dispensable for survival of normal cells.

(C) Ectopic expression of UACA inhibits the secretion of Par-4. MEFs from Par-4<sup>−/−</sup> mice were co-transfected with Par-4 expression construct plus vector control, Par-4 and UACA expression construct (1:1 ratio), or with vector control. Transfected populations were selected, confirmed for stable expression of Par-4 and UACA using whole-cell lysates, and then secretion of Par-4 in the CM was examined by Western blot analysis.

(D) UACA binding to Par-4 is essential in order to prevent Par-4 secretion. Par-4<sup>+/+</sup> MEF cells were transfected with expression constructs for Red Fluorescent Protein (RFP; vector), RFP-tagged UACA, or RFP-tagged UACA deletion mutants, 1-630 (which binds to Par-4) or 631-1413 (which does not bind to Par-4) (Burikhanov et al., 2013). Transfected populations were selected, confirmed for stable expression of UACA or UACA-mutants using whole-cell lysates, and secretion of Par-4 in their CM was determined by Western blot analysis.

(E) Illustration of full length UACA and deletion mutants of UACA subcloned in the CMV-promoter based expression vector pCB6. Full length UACA or deletion mutants 1-630aa or 631-1413aa (with in-frame RFP tagged at its C-terminus) are shown. The sizes of full-length UACA and mutant UACA fragments subcloned into pDsRed vector are indicated.
SUPPLEMENTAL DATA

FIGURE S3

A

p53-binding motif:
Consensus: 5'-PuPuPuCu(T/A)G,G,PuPuPyPy [0-13] PuPuPuPuCu(T/A)G,G,PuPuPyPy-9'
UACA: 5'-GGACGTTGCCGCGC-14CCGATGGTC-3'
Mutant UACA: 5'-GGATGTTCCGGCGCCDCAATGTC-3'
p21 (#1) 5'-GAACCATGTTCCCAACATGATTTGC-3' (distal site)
p21 (#2) 5'-AGACCATGTTCCCAACATGATTTGC-3' (proximal site)

![Diagram of UACA and mutant UACA with coordinates and sequences]

B

![Graph showing relative levels of UACARNA, p53, and actin]

C

![Image showing gel electrophoresis with bands at 200, 132, and 100 bp for Ad-GFP-p53, Ad-GFP, and input]
**SUPPLEMENTAL DATA**

Figure S3 Related to Figures 3 and 4. **UACA contains a p53 consensus binding motif and p53 down-regulates UACA by directly binding to UACA DNA.**

(A) **Comparison of the p53 response elements in UACA and p21, and location of wild type p53 binding motif and mutations introduced in UACA.** The consensus sequence for p53-binding DNA motif is shown along with the p53-binding motifs in UACA and p21. The indicated mutations were introduced at C4, G7, C14 and G17 into the p53-binding motif of UACA. P21 has two p53-binding motifs (#1 and #2) (El-Deiry et al., 1995). The nucleotides in the p53-binding motif of p21 that deviate from the p53 consensus binding motif are underlined. Mutant-UACA with the altered p53-binding site was generated by PCR using mutant-UACA primers and wild type UACA DNA as template, and fidelity of the UACA constructs was confirmed by Retrogen Inc., CA.

(B) **P53 inhibits the expression of UACA RNA.** Cells were infected with either control GFP-producing adenovirus or GFP-tagged p53-producing adenovirus for 24 h. Total RNA was isolated from each of the samples and subjected to qRT-PCR (upper panel), or whole cell lysates were subjected to Western blot analysis (lower panel).

(C) **P53 binds to its consensus binding motif in UACA.** A putative p53 binding motif (5’-GGAC_{C4}GT_{G7}CCCGGCC_{C14}CCG_{G17}CGTC-3’; where C4 and G7 have been previously shown to be critical) (El-Deiry et al., 1992) is present in the coding region of UACA. P53-deficient cells were infected with GFP-tagged p53- or GFP-producing adenovirus and subjected to ChIP analysis with p53 antibody (Ab) or control rabbit Ab, and immunoprecipitated DNA fragments were analyzed by PCR with primers flanking the p53 binding site.
Figure S4 Related to Figure 4. P53 regulates UACA expression via its binding motif in an NF-κB activity-independent manner. IKKβ⁻/⁻ MEFs (right and bottom panels) or wild type MEFs (left panel), as controls, were co-transfected with the indicated expression constructs. Whole-cell lysates were subjected to Western blot analysis for UACA, p53 or actin (top panels) or RNA was prepared from the cells and subjected to reverse transcriptase PCR for UACA and 18S rRNA (bottom panel). Wild type (wt) UACA contained wild type p53-binding motif 5'-GGA<sub>C4</sub>GT<sub>G7</sub>CCCGGC<sub>C14</sub>C<sub>G17</sub>CGTC-3', and mutant (m) UACA contained mutations 5'-GGAT<sub>T4</sub>GTA<sub>7</sub>CCCGGC<sub>G14</sub>CCA<sub>17</sub>CGTC-3' in the p53 binding sequence.
**SUPPLEMENTAL DATA**

**SUPPLEMENTAL EXPERIMENTAL PROCEDURES**

**Plasmids and chemical reagents**

z-VAD-fmk was from BioVision, and PS-1145, Doxorubicin and BFA were from Sigma Chemicals. UACA-constructs were described previously (Burikhanov et al., 2013). The p53 constructs were from Wafik El-Deiry (Penn State University, PA). Transfections were performed using Lipofectamine and Plus reagents (Invitrogen).

**Antibodies and siRNA duplexes**

The polyclonal antibodies for UACA were from Bethyl Laboratories and Abcam. Par-4 (R332), Col1A1 (H-197), pan-cytokeratin (C11), p53 (FL-393), p21/WAF1 (F5), PTEN (FL-403) and rabbit IgG antibodies were from Santa Cruz Biotechnology, Inc. The mouse monoclonal antibody for p53 (1C12 mAb), and cleaved caspase-3 antibody (D175) were from Cell Signaling, and that for β-actin was from Sigma Chemical Corp. The control siRNA and pools of siRNA for UACA were from Dharmaco and SantaCruz Biotechnology, Inc.

**Co-immunoprecipitation and Western blot analysis**

Protein extracted from cell lysates was filtered, pre-cleared with 25 µl (bed volume) of protein G-Sepharose beads and immunoprecipitated with 1 µg of respective antibodies. The eluted proteins were resolved by SDS-PAGE, and subjected to Western blot analysis as described (Goswami et al., 2008).

**Quantitative Real Time-PCR analysis**

Approximately 1x10^6 cells were infected with GFP control or GFP-tagged p53-adenovirus for 24 hours, and total RNA was extracted using Trizol reagent. Quantitative Real Time-PCR (qRT-PCR) was performed using SuperScript First-Strand Synthesis System (Invitrogen) using oligo (dT) primers for RT. Brilliant II SYBR Green Master Mix (Agilent Technologies) was used for subsequent qPCR. Primers used were as follows: Forward: 5'-cgc cc tag agg tga aat tct-3' and reverse: 5'-cga acc tcc gac ttt cgt tct-3' for 18S RNA, and forward primer: 5'-ggc gga gaa cga taa gtt gac ttc gac taa-3', and reverse primer: 5'-cat gtt tgt cgg gag cta caa ac-3' for qPCR of human UACA.

**Reverse transcriptase-PCR analysis**

For the reverse-transcriptase PCR experiment with co-transfection, approximately 1.5x10^6 IKKβ^-/- cells were co-transfected with either vector, wild-type(wt) UACA, or mutant(m) UACA construct with or without p53 plasmid for 72 hours, and total RNA was extracted using RNeasy mini kit (Qiagen). Reverse transcription was performed to synthesize cDNA with random hexamers using SuperScript III First-Strand Synthesis System (Invitrogen), and was followed by PCR. Primers used were as follows: Forward primer: 5'- gta acc cgt tga acc cca tt -3' and reverse primer: 5'- cca tcc aat cgg tag tag cg -3' for 18S RNA.

Forward primer: 5'-aag agc ctc aag tcc cgc c -3', and reverse primer: 5'- ccc ccc ttg ctt ctt-3' for pCB6“ human UACA wild type or mutant constructs. The products were subjected to DNA gel electrophoresis.
**SUPPLEMENTAL DATA**

**ChIP analysis**
ChIP assays for endogenous p53 binding to the consensus binding site in UACA were performed by treating 2x10^6 HEL cells with either DMSO vehicle or with Nutlin-3a for 24 h, and 1x10^6 cells were used per ChIP reaction in each treatment group. Alternatively, for ChIP analysis with ectopic p53 expression, 1x10^6 cells infected with either GFP control or GFP-tagged p53 adenovirus were used per ChIP assay. ChIP analysis was performed using the ChIP kit from Millipore according to the instructions provided by the manufacturer. Briefly, the proteins and DNA were cross-linked with 1% formaldehyde, lysed, and the DNA was sheared into 200-800 bp fragments. Proteins linked to the DNA were immunoprecipitated with appropriate antibodies (using rabbit IgG or no antibody as control). Subsequently, immune complexes were eluted from the beads, protein-DNA crosslinks were reversed, and DNA was isolated after phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. DNA fragments were amplified by PCR using the following primers.

UACA: Forward primer 5’-gtc tac tcc ttg cgc gct gg-3’ and reverse primer 5’-gcg gcg cca gac gac cca gac gac-3’
p21: Forward primer 5’-ctg gac tgg gca ctc ttg tc- 3’ and reverse primer 5’-ctc cta cca tcc cct tcc tc- 3’
GAPDH: Forward primer 5’ – atg gtt gcc act ggg gat ct- 3’ and reverse primer 5’ – tgc caa agc cta ggg gaa ga- 3’

**Apoptosis assays**
Apoptotic nuclei were identified by immunocytochemical (ICC) analysis for active caspase-3, or by 4, 6-diamidino-2-phenylindole (DAPI) staining. A total of three independent experiments were performed; and approximately 500 cells were scored in each experiment for apoptosis under a fluorescent microscope.

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**SUPPLEMENTAL REFERENCES**


