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Cancer Research

p53 Binds to and Is Required for the Repression of *Arf* Tumor Suppressor by HDAC and Polycomb

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Abstract

The expression of tumor suppressor *Arf* is tightly repressed during normal cell growth at a young age and is activated by oncogenic insults, and during aging, results in p53 activation and cell-cycle arrest to prevent hyperproliferation. The mechanisms of both transcriptional repression and activation of *Arf* are not understood. We show that p53 binds to and represses *Arf* expression and that this repression requires the function of both histone deacetylases (HDAC) and polycomb group (PcG) proteins. Inactivation of *p53* leads to increased *Arf* transcription in both mouse embryonic fibroblasts (MEF) cultured *in vitro* and in tissues and organs of *p53* null mice. Activation of endogenous p53 enhances *Arf* repression, and reintroduction of p53 back into *p53* null MEFs restores *Arf* repression. Both DNA binding and transactivation activities of p53 are required for *Arf* repression. We show that p53 is required for both HDAC and PcG to repress *Arf* expression. Bindings of both HDAC and PcG to *Arf* are disrupted by inactivation of p53 and can be restored in *p53* null MEFs by the reintroduction of wild-type, but not mutant, p53. These results indicate that p53 recruits both HDAC and PcG to *Arf* locus to repress its expression, and this repression constitutes a second feedback loop in p53 regulation. *Cancer Res; 71(7); 2781–92.* ©*2011 AACR.*

Introduction

Transcription factor p53 mediates cellular response to a wide range of genotoxic and growth stresses including DNA damage and oncogenic insults (1). Activated p53 increases the expression of numerous genes and elicits 3 distinct types of cellular outcomes—temporary cell-cycle arrest, permanent cell senescence, and apoptotic cell death—to prevent damaged or stressed cells from continuing proliferation (2). It is generally believed that escape from the p53-mediated checkpoint pathway is a necessary step for the development of most, if not all, types of tumors (3, 4).

Much has been learned on the function of p53 in both senescence and apoptosis (5). Equally important is the regulation of p53 in causing temporary cell-cycle arrest. Unlike senescence or apoptosis, both of which are irreversible, a mechanism to inhibit activated p53 is critically important in releasing temporarily arrested cells to resume the cell cycle once the damage is repaired or stress is relieved. One such mechanism to reversibly regulate p53 is the feedback inhibition loop in which p53 activates the expression of its principle inhibitor (6–8), MDM2, which binds to and inhibits the function of p53 by both repressing the transactivating activity of p53 (9, 10), as well as targeting p53 for ubiquitin-mediated degradation (11–13).

The *ARF-p16* locus, which is altered in an estimated 30% to 40% of human tumors, encodes 2 distinct tumor suppressor gene products, $p16^{INK4a}$ and $p14^{ARF}$ (p19 in mouse), via the use of separate promoters and alternative reading frames (14–16). Whereas $p16^{INK4a}$ binds to and inhibits cyclin D-dependent CDK4 and CDK6 to retain the growth suppressive activity of Rb family proteins (17), ARF binds to and antagonizes the activity of MDM2, thereby stabilizing and activating p53 (18–20). The *ARF* gene is expressed at a low level in normally growing young cells. Previous studies have linked 2 histone-modifying complexes, histone deacetylases (HDAC; refs. 21–23) and polycomb proteins (PcG; refs. 24–30), to the repression of *Arf* expression. How these 2 histone-modifying complexes, which do not recognize a specific DNA sequence, are recruited to *Arf* locus is not known.

ARF is activated by various oncogenic insults or during cell aging, leading to the notion that ARF mediates an oncogene checkpoint pathway to prevent oncogenic-stimulated cells from hyperproliferating (15, 31). The potent activity of ARF in binding to and inhibiting the function of MDM2 raises the question of how p53 is reversibly regulated in oncogenically

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insulted cells where ARF, if continuously expressed, would prevent MDM2 from inhibiting p53 and thus disrupt the MDM2–p53 feedback regulatory loop. It was noted early on that in both human (19, 32) and mouse cells (33, 34), *ARF* expression exhibits a strong inverse correlation with the functional status of p53, suggesting a possible feedback repression of *ARF* expression by p53. The molecular mechanism underlying this feedback regulation is unknown and is the focus of this study.

Materials and Methods

Cell culture, Western analysis, and antibodies

Mouse embryonic fibroblasts (MEF) and 293T cells were cultured in DMEM (Dulbecco's modified Eagle's medium) with 10% FBS. Cells were lysed with RIPA (radioimmunoprecipitation assay) buffer. Antibodies to Bmi1 (F6; Upstate), Ring1B (ab-3832; Abcam), Suz12 (ab-12073; Abcam), Ezh2 (ab-3748; Abcam), HDAC1 (ab-7028, Abcam); acetyl-histone H4 (06-598; Upstate), p53 (FL-393X; Santa Cruz), $p19^{Arf}$ (ab-80; Abcam), mouse p16 (M-156; Santa Cruz), 3m-H3K27 (ab-6002; Abcam), E2F3 (sc-878X; Santa Cruz), tubulin (Ab-2 DM1A; Neomarkers), normal mouse IgG (Neomarkers), normal rabbit IgG (Neomarkers), and actin (C-11; Santa Cruz) were purchased commercially.

Retroviral procedures

The retroviral vector expressing mouse Bmi1 and microRNA targeting 3′-untranslated region (UTR) of mouse p53 was provided by Dr. Ned Sharpless and Yizhou He, respectively. Human *p53* cDNA was cloned into pBabe-puro retrovirus vector, and point mutations were made by site-directed mutagenesis and verified by DNA sequencing. Retroviruses encoding short hairpin RNA (shRNA) silencing *mBmi1*, *mE2f3*, *mHdac1*, and *mHdac2* were constructed by ligating respective oligonucleotides (see details in Supplementary Materials and Methods) into a PMKO-puro vector. Detailed experimental procedures for retroviral production and infection are described in the work of Kotake and colleagues (30).

Quantitative reverse-transcriptase real-time PCR

Detailed protocol has been described in the work of Kotake and colleagues (30). Sequences of PCR primers are described in Supplementary Materials and Methods. Mean values and SDs were calculated from triplicates of 3 independent repeats.

ChIP assay

Chromatin immunoprecipitation (ChIP) analysis was performed as described in the work of Kotake and colleagues (30). PCR was performed using Platinum Taq polymerase (Invitrogen) and primers on mouse *Arf* locus (see more details in Supplementary Materials and Method). For ChIP-Q-PCR, purified DNA was added to a quantitative reverse-transcriptase real-time PCR (Q-RT-PCR) mixture that contained 1× SYBR Green PCR master mix and 150 nmol/L gene-specific primers. Assays were performed in triplicate on a 7900 HT sequence detection system. Mean values and SDs were calculated from triplicates of 3 different repeats.

Results

p53 represses *Arf* expression *in vivo*

Confirming previous observations (33, 35), we found that the steady-state level of Arf protein is significantly increased in *p53*-deficient MEFs (Fig. 1A). We also observed a clear increase of p16 protein level in $p53^{-/-}$ MEFs, which could be caused by the decrease of *p21* expression and then a reduction in function of the Rb pathway, which collaborates with polycomb repressive complex (PRC) to repress *p16* gene transcription in a feedback loop (30). A demonstration of *Arf* repression by p53 *in vivo*, however, has been lacking. Therefore, we dissected 3 pairs of age-matched (1 pair at 5 weeks of age and 2 at 8 weeks of age) wild-type (WT) and *p53* null mice and determined the *Arf* expression in 8 different organs/tissues. This study demonstrated that *Arf* expression was significantly increased by *p53* loss in 4 organs (muscle, kidney, heart, and lung), moderately in 2 (liver and spleen) and unchanged in 2 (thymus and testis; Fig. 1B). This result provides the first evidence demonstrating p53-dependent repression of *Arf in vivo*.

To exclude the possibility that *Arf* accumulation in *p53* null MEFs was caused indirectly by other potential mutations and/or adaptive changes accumulated during multiple rounds of cell division, we transduced WT MEFs with a retrovirus expressing the type 16 papilloma virus–encoded E6 oncoprotein that binds to and targets the degradation of p53, or a microRNA retrovirus targeting 3′-UTR of mouse p53. Ectopic expression of E6 or mip53 resulted in a detectable increase of Arf protein and mRNA as early as 2 days after viral transduction and a continual increase of Arf (Fig. 1C and Supplementary Fig. SA and B). This result supports a direct role of p53 in the repression of *Arf* transcription and also suggests a continuous need of p53 to maintain the repression.

Nutlins are a group of small compounds that can bind MDM2 in the p53-binding pocket and disrupt the p53–MDM2 interaction, leading to p53 activation (36). We treated WT MEFs at passage 2 with Nutlin-3 (10 μmol/L) or dimethyl sulfoxide (DMSO) for over 24 hours and then examined *Arf* expression. Confirming the activation of p53, Nutlin-3 increased *p21* mRNA in WT MEFs, but had no effect on *p21* level in $p53^{-/-}$ MEFs. Nutlin-3 treatment enhanced *Arf* repression in WT MEFs within 24 hours, resulting in a time-dependent decrease of *Arf* expression by 60%, but no effect on *Arf* level in $p53^{-/-}$ MEFs despite the much higher *Arf* transcription (Fig. 1D). We thus conclude that p53 represses *Arf* at a level of transcriptional regulation through a mechanism that requires a direct and continuous role of p53.

p53 binds to *Arf* locus

That p53 is directly involved in *Arf* repression led us to determine whether p53 binds to the *Arf* locus. We performed ChIP analysis using a panel of 35 pairs of oligonucleotide primers that span 4-kb upstream and 4-kb downstream of the transcription start site of mouse *Arf*. We detected direct p53 binding to a region immediately upstream and downstream of

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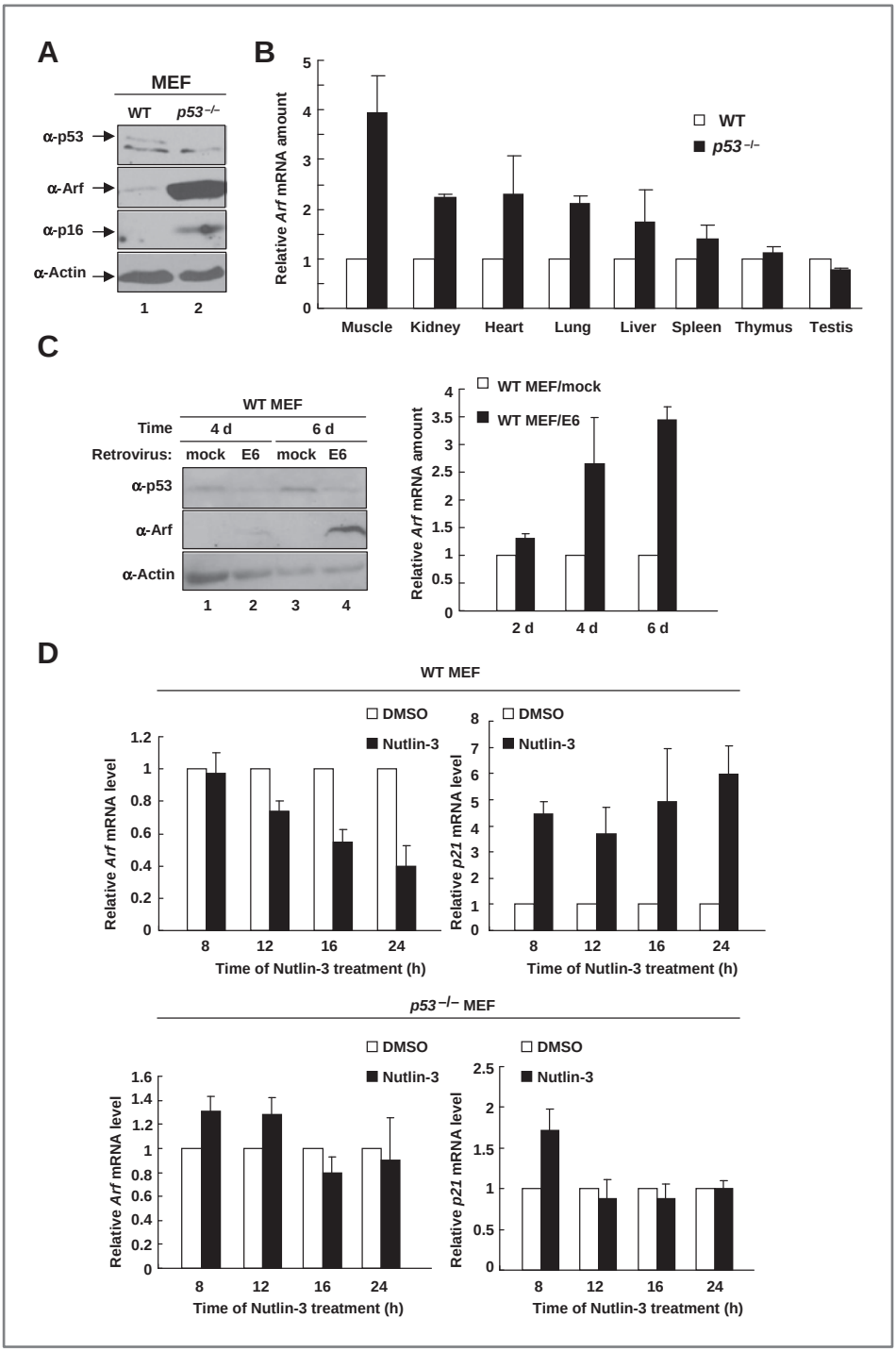

Figure 1. p53 represses *Arf* expression. A, the steady-state levels of Arf and p16 proteins were determined in WT and $p53^{-/-}$ MEFs at passage 5 (p5) by immunoblotting. B, three pairs of age-matched WT and $p53^{-/-}$ mice were dissected and total RNA was extracted from 8 different organ/tissues. The level of *Arf* mRNA was determined by Q-RT-PCR. C, WT MEFs (p2) were infected with mock or E6-expessing retrovirus and selected by G418 treatment. The level of p53 and Arf proteins or mRNA was determined by immunoblotting or Q-RT-PCR. D, WT (p2) and $p53^{-/-}$ MEFs were treated with 1% DMSO or 10 μmol/L Nutlin-3 and the levels of *Arf* and *p21* mRNA were determined by Q-RT-PCR.

exon 1β (*amplicons a*, *b*, and *c* for regular PCR; corresponding *amplicons A*, *B*, and *C* for Q-PCR) of *Arf* (Fig. 2A). In contrast, there was very little binding of p53 to the *p16* promoter or no p53 binding was detected by using $p53^{-/-}$ MEF as negative control in ChIP analysis. These results demonstrate that p53 affects the expression of *Arf* and *p16* differently and indicate a direct role of p53 in the repression of *Arf* expression.

p53-mediated repression of *Arf* needs both transactivation and DNA binding activity

To provide further evidence supporting a direct role of p53 in *Arf* repression, we examined 4 p53 mutants in *Arf* repression, including 2 well-characterized p53 hot spot mutants—R175H, which grossly disrupts protein conformation of p53 and R273H, which retains native conformation of p53 but loses contact

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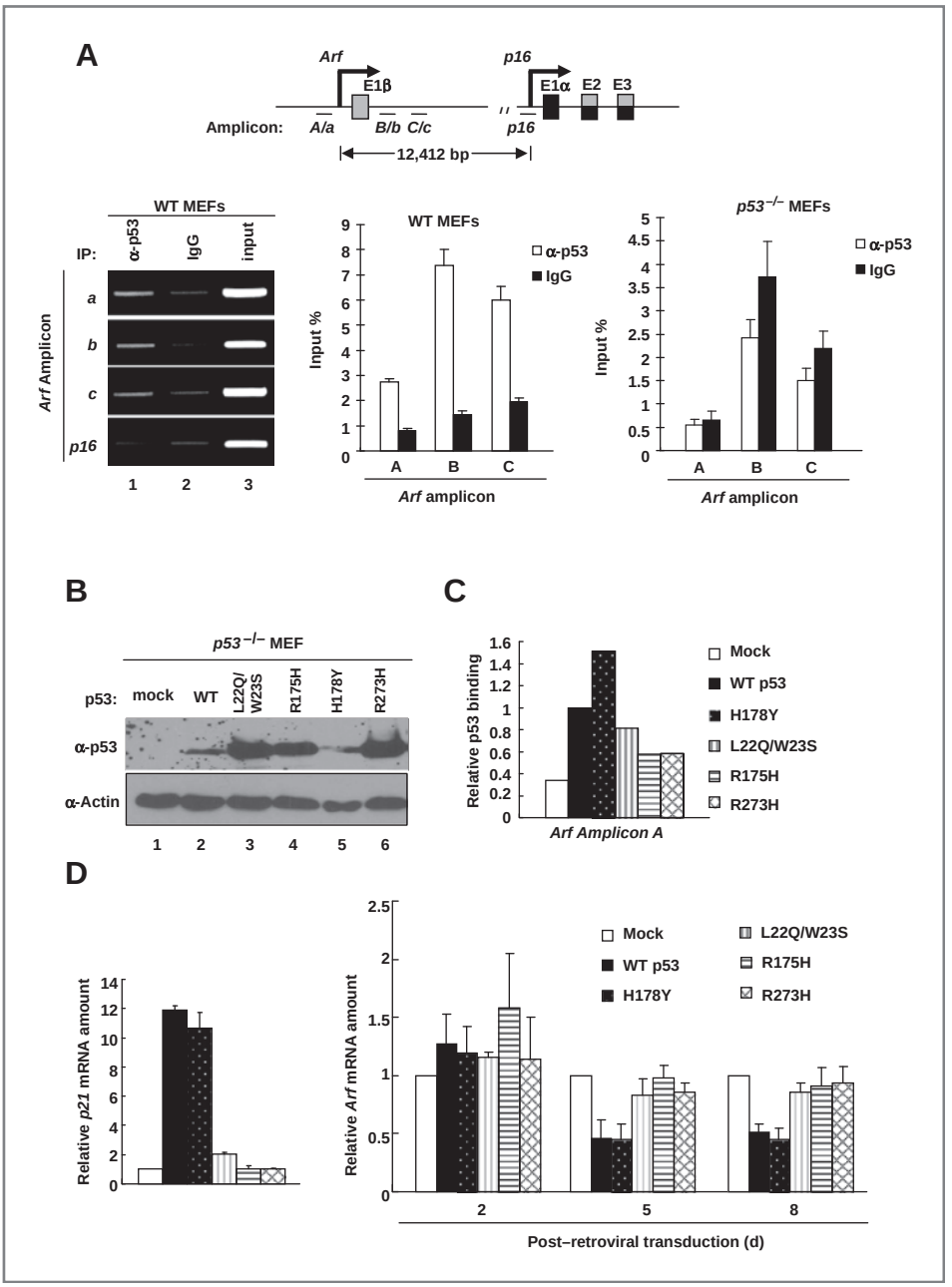

Figure 2. Both transactivation and DNA binding activity of p53 are required for p53 to bind and repress *Arf*. A, WT p2 and $p53^{-/-}$ p3 MEFs were analyzed for p53 binding on *Arf* and *p16* locus. The amount of DNA immunoprecipitated by p53 or rabbit IgG was expressed relative to the percentage of input DNA. B, $p53^{-/-}$ MEFs were infected with mock or WT p53 and mutant p53 retrovirus. Cells were selected by puromycin treatment, collected at indicated time after infection, and the expression levels of p53 were determined by immunoblotting. C, cells in B collected 8 days after infection were analyzed for binding of p53 on *Arf* locus by ChIP-Q-PCR. D, *p21* mRNA levels of cells in B were determined 2 days after infection by Q-RT-PCR, and cells collected at different times after infection were analyzed for *Arf* mRNA levels by Q-RT-PCR.

with DNA (see a recent review on the genetic and biochemical properties of different p53 mutants; ref. 37). In addition, we also examined a double mutant—L22Q/W23S—which disrupts transcriptional activity of p53, as well as the binding with Mdm2 (38) and a hyperactive p53 mutant (H178Y) that could rescue the inactivated function of a common mutation, *G245S* (39). $p53^{-/-}$ MEFs were transduced with a retrovirus expressing the WT and individual mutants of p53. As expected, the 3 functional inactivation mutants ($p53^{L22Q/W23S}$, $p53^{R175H}$ and $p53^{R273H}$) were expressed at high levels, whereas cells could only tolerate a much lower level expression of both WT p53 and hyperactive $p53^{H178Y}$ mutant (Fig. 2B). The activity of the WT and individual p53 mutants was functionally verified by examining the expression of *p21* (Fig. 2D).

ChIP and Q-RT-PCR assay showed that $p53^{H178Y}$ bound to *Arf* stronger than the WT p53 and $p53^{L22Q/W23S}$ exhibited decreased *Arf* binding. Both $p53^{R175H}$ and $p53^{R273H}$, however, bound very weakly to *Arf* (Fig. 2C). We then determined whether these p53 mutants could restore *Arf* repression in *p53* null MEFs. Starting 5 days after viral transduction, both WT and the hyperactive $p53^{H178Y}$ mutant reduced the *Arf* mRNA level by 50% (Fig. 2D). In contrast, ectopic expression of $p53^{L22Q/W23S}$, $p53^{R175H}$, and $p53^{R273H}$ at very high levels had little effect in restoring the repression of *Arf*. Together, these

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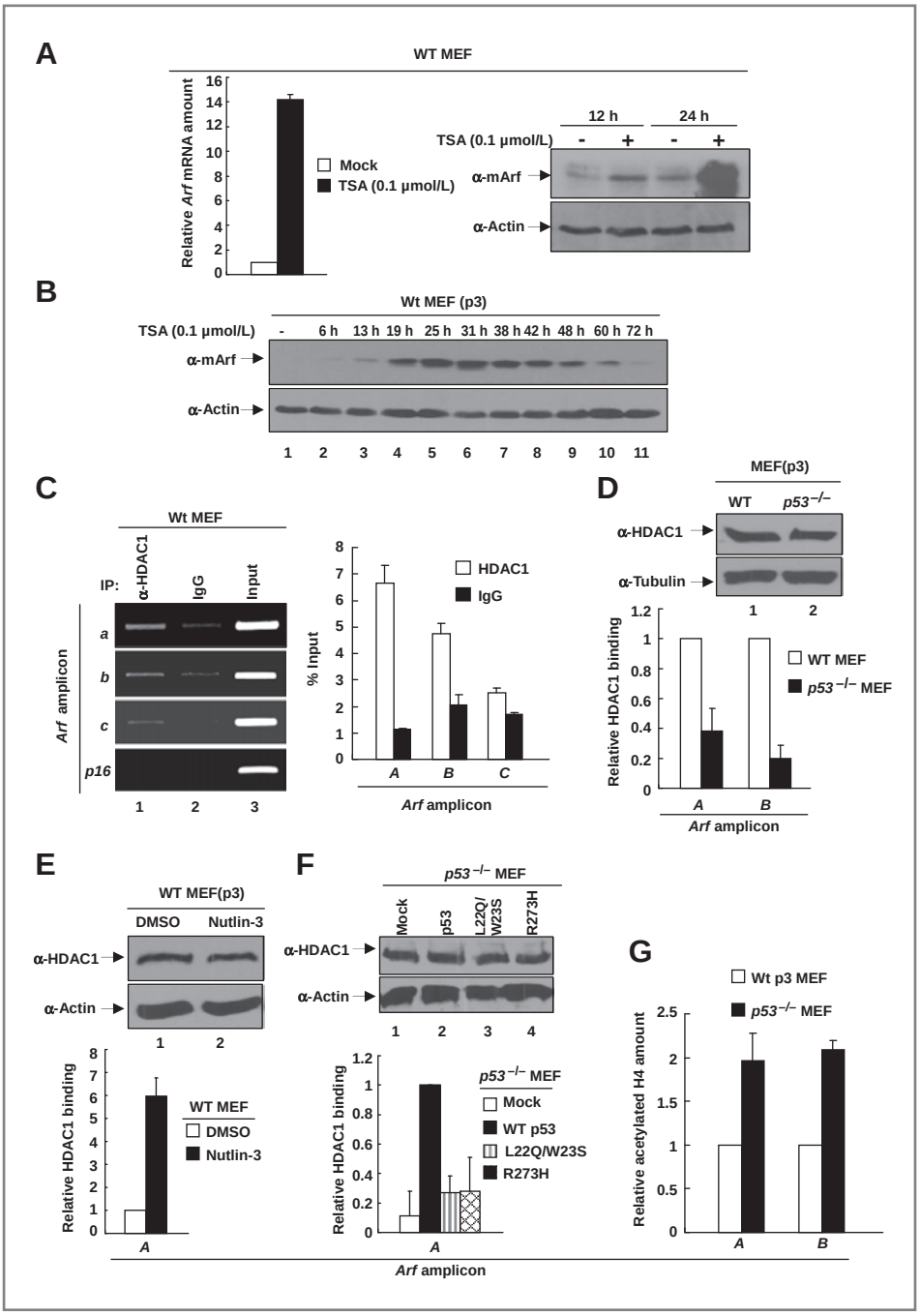

Figure 3. HDAC1 binds to and represses *Arf* in a p53-dependent manner. A, WT p3 MEFs were treated with 0.1 μmol/L TSA for 12 or 24 hours. The levels of Arf protein or mRNA were determined by immunoblotting or Q-RT-PCR. B, WT p3 MEFs were treated with 0.1 μmol/L TSA for 0 to 72 hours. Cell pellets were collected at indicated time and steady-state level of protein was determined by immunoblotting. C, binding of HDAC1 to *Arf* locus was examined in WT p2 MEFs by ChIP analysis. The amount of DNA immunoprecipitated by HDAC1 or rabbit IgG was expressed relative to the percentage of input DNA. D and G, binding of HDAC1 on *Arf* and amount of acetylated histone H4 on *Arf* were quantified in WT and $p53^{-/-}$ MEFs by ChIP-Q-PCR. E, WT p3 MEFs were treated with 1% DMSO or 10 μmol/L Nutlin-3 and cells were collected after 16 hours. The binding of HDAC1 on *Arf* was determined by ChIP-Q-PCR with indicated primers. F, $p53^{-/-}$ MEFs were infected with mock or WT and mutant p53 retrovirus. Cells were selected by puromycin treatment, collected at 5 days postinfection, and analyzed for HDAC1 binding to *Arf* by ChIP-Q-PCR.

results demonstrate that p53 represses *Arf* by directly binding to the *Arf* locus and that both transactivity and DNA binding of p53 are essential to repress *Arf* expression.

HDAC binds to and represses *Arf* and is recruited to *mArf* in a p53-dependent manner

HDACs associate with many transcriptional repressive complexes and have also been reported to participate in the transcriptional repression of both human and mouse *ARF* genes (22, 23). To determine whether HDACs are involved in p53-mediated repression of *Arf* transcription, we treated WT MEFs with trichostatin A (TSA), an inhibitor of class I/II HDACs. A low concentration of TSA (0.1 μmol/L) increased mouse *Arf* mRNA level by more than 15-fold within 24 hours (Fig. 3A). TSA treatment resulted in a detectable Arf protein increase as early as 6 hours, peaking around 25 hours and lasting to as long as 60 hours (Fig. 3B). These results demonstrate a potent role of HDACs in the repression of mouse *Arf* transcription.

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We next tested the binding of HDAC1 on *Arf* in WT MEFs. ChIP showed that HDAC1 directly binds to a region immediately upstream of the transcription starting site of *Arf*, whereas no binding of HDAC1 was detected on the nearby *p16* locus (Fig. 3C). Importantly, the binding of HDAC1 to mouse *Arf* was greatly decreased, by more than 60% (for amplicon A) to 80% (amplicon B), in $p53^{-/-}$ MEFs (Fig. 3D) or in WT MEFs with p53 knocked down by microRNA (Supplementary Fig. SC), providing the first evidence linking HDAC-mediated repression of *Arf* transcription to the function of p53. Supporting a functional dependency of deacetylation of *Arf* promoter on p53 function, activation of p53 by Nutlin-3 in WT MEFs resulted in a 6-fold increase of HDAC1 binding to *Arf* (Fig. 3E) and reintroduction of WT p53, but not $p53^{L22Q/W23S}$ and $p53^{R273H}$, back into $p53^{-/-}$ MEFs restored the binding of HDAC1 to the *Arf* locus (Fig. 3F). Finally, we showed that H4 acetylation of the *Arf* promoter in $p53^{-/-}$ MEFs was 2-fold higher than that in WT MEFs (Fig. 3G). Together, these results demonstrate that HDAC1 directly binds to and deacetylates *Arf* in a p53-dependent manner.

p53 is required for PRC to bind and repress *Arf*

Previous studies have shown that oncogene *Bmi1* promotes cell proliferation and extends the life span of fibroblasts, in part, through repressing both $p16^{Ink4a}$ and $p19^{Arf}$ expression (24, 25). More recently, PcG proteins have been shown to directly bind and repress both *p16* and *ARF* in mouse cells (29, 30). To determine the functional interplay between p53 and PcG in repressing *Arf* expression, we transduced both WT and $p53^{-/-}$ MEFs with a retrovirus expressing the *Bmi1* gene and determined *Arf* expression. Nine days after retrovirus transduction (passage 5), both the steady-state level of the Arf protein and mRNA were decreased substantially in Bmi1-overexpressing WT MEFs (Fig. 4A). In contrast, although *Arf* is expressed at a much higher level in $p53^{-/-}$ MEFs, overexpression of Bmi1 did not significantly affect either Arf protein or mRNA level. To determine the specificity of the functional dependency of Bmi1-mediated repression of *Arf* on p53, we examined the expression of 2 classical Bmi1-repressive targets—*HoxA9* and *HoxC13* (40). Ectopic expression of Bmi1 was capable of repressing both *HoxA9* and *HoxC13* genes regardless of the *p53* status (Fig. 4B). Together, these results demonstrate a specific functional dependency of Bmi1-mediated repression of *Arf* transcription on p53.

Bracken and colleagues previously found that the PRC can bind directly to the mouse *Arf* locus to repress its expression (29). To search for the mechanism of the p53 dependency of *Arf* repression by Bmi1, we examined the binding of Bmi1 and Ring1B, another component of PRC, to the *Arf* locus in WT and $p53^{-/-}$ MEFs. Notably, the bindings of both Bmi1 and Ring1B to the *Arf* locus were greatly decreased in $p53^{-/-}$ MEFs (Fig. 4C). Confirming the p53 dependency, binding of Ring1B to the *Arf* locus in WT MEFs was also greatly reduced after transduction with an E6-expressing or p53-targeting microRNA retrovirus (Fig. 4D and Supplementary Fig. SC) and conversely increased in WT MEFs when p53 was activated by Nutlin-3 (Fig. 4E). Together, these results demonstrate a requirement of p53 for the binding of PRC1 to *Arf* locus.

Previous studies on *Hox* gene silencing by PRC suggest a sequential model whereby PRC2-mediated H3K27 trimethylation facilitates the recruitment of PRC1, which causes H2A-K119 ubiquitylation to repress *Hox* gene expression (41, 42). We further analyzed the binding of PRC2 and H3K27 trimethylation on *Arf* and quantification showed a dramatic decrease of this repressive marker in $p53^{-/-}$ MEFs to less than 7% (Fig. 4F). Likewise, the binding of 2 components of PRC2, Ezh2 and Suz12, to the *Arf* locus were also substantially reduced in $p53^{-/-}$ MEFs to 29% and 6.7% for Ezh2 and to 43% and 18% for Suz12 of WT MEFs at sites A and C, respectively (Fig. 4F). Hence, the p53 function is required for both PRC1 and PRC2 to bind to the *Arf* locus.

Both transactivity and DNA binding activities of p53 are required for PRC1 binding to *Arf*

To further demonstrate the requirement of p53 in facilitating the binding of PRC1 to the *Arf* locus, we reintroduced p53 into $p53^{-/-}$ MEFs which partially restored the repression of both Arf protein and mRNA (Fig. 5A and B). The binding of Ring1B to *Arf* was also restored when p53 was ectopically expressed, resulting in a 2- to 3-fold and 4- to 7-fold increase of Ring1B binding to *Arf* in $p53^{-/-}$ MEFs after adenovirus- and retrovirus-mediated p53 expression, respectively (Fig. 5A and B). We then determined whether mutant p53 restored PcG binding to *Arf*. Both WT p53 and the hyperactive $p53^{H178Y}$ mutant, but not 3 inactivated mutants, restored Ring1B binding to *Arf*, leading to a 2.4- and 1.9-fold increase, respectively (Fig. 5C). Together these results demonstrate that both the trans-activating and DNA binding activities of p53 are required for the binding of PcG to *Arf*.

HDAC function is required for PcG to bind mouse *Arf*

Given that p53 is required for the binding of both HDAC and PcG complexes to the *Arf* locus and that HDAC has been reported to be in the same repressive complex with and facilitates the repressive function of PcG (43, 44), we next determined the requirement of HDAC function for PcG binding on *Arf* by 2 different approaches, pharmacologic inhibition of HDAC activity and shRNA-mediated depletion of HDACs. Treatment of WT MEFs with TSA and another pharmacologic inhibitor of HDAC, sodium butyrate (NaB), both significantly increase *Arf* expression, but neither affected Ring1B level (Fig. 6B). Inhibition of HDAC by either TSA or NaB increased H4 acetylation, yet decreased Ring1B binding and H3K27 trimethylation on *Arf* promoter (Fig. 6A and C).

We then used shRNA to knockdown *HDAC1* and *HDAC2* in WT MEFs. Although only a partial depletion was achieved, we observed a decrease of Bmi1 binding to *Arf* and am increase of *Arf* expression (Fig. 6D). These results demonstrate that the activity of HDAC is required for PcG to bind to and repress the expression of *Arf*, and both HDAC1 and HDAC2 contribute to this inhibition.

HDAC and PcG function are required for p53 to repress *Arf* expression

The functional dependency of both HDAC and PcG on p53 in the repression of *Arf* expression led us to determine whether

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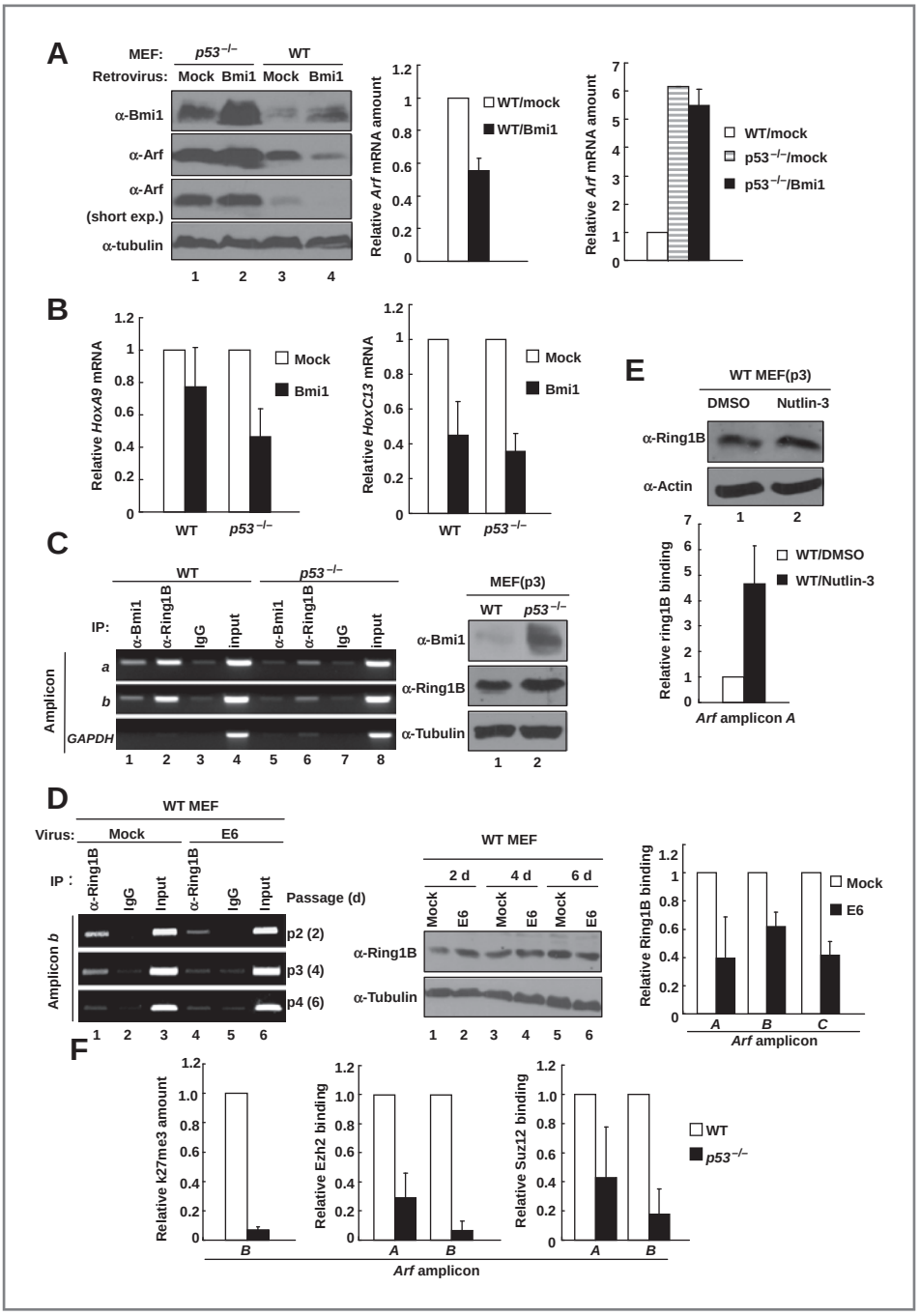

Figure 4. p53 is required for PRC to bind and repress *Arf*. A and B, p2 WT and $p53^{-/-}$ MEFs were infected with mock or Bmi1-expressing retrovirus. Nine days after infection, MEFs were collected and the protein levels of Bmi1 and Arf were determined by immunoblotting; the mRNA levels of *Arf* (A), *HoxA9*, and *HoxC13* (B) were determined by Q-RT-PCR. C, binding of Bmi1 and Ring1B to *Arf* locus was tested in WT p2 and $p53^{-/-}$ MEFs by ChIP. D, WT p2 MEFs were infected with mock or E6 retrovirus and G418 selected. Cells were collected at indicated time after infection and analyzed for Ring1B binding on *Arf*. E, WT p3 MEFs were treated with 1% DMSO or 10 μmol/L Nutlin-3, and cells were collected after 16 hours. The binding of Ring1B on *Arf* was determined by ChIP-Q-PCR. F, ChIP-Q-PCR was used to determine the trimethylation of H3K27 and binding of Ezh2 and Suz12 on *Arf* locus in WT p3 and $p53^{-/-}$ MEFs. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

these 2 histone-modifying enzymes are required for p53 to repress *Arf* expression. To this end, we inhibited endogenous HDAC with TSA in WT MEFs and then determined whether activation of endogenous p53 by Nutlin can still repress *Arf*. While activation of p53 by Nutlin-3 resulted in a greater than 50% decrease of *Arf* expression in control MEFs, it had no detectable effect on *Arf* expression when the HDACs were inhibited by TSA, even though the *Arf* mRNA level was elevated by as much as more than 16-fold in the TSA-treated cells (Fig. 7A). As a control for p53 activation, inhibition of HDACs by TSA did not affect the increase of *p21* expression by Nutlin-activated p53.

To determine whether the function of PcG is required for p53 to repress *Arf* expression, we knocked down the expression of *Bmi1* in WT MEFs and examined the effect of *Bmi1* silencing on *Arf* repression by Nutlin-activated p53. All 3 shBmi1 viruses efficiently reduced the expression of *Bmi1* (Fig. 7B), and none of them affected the activation of *p21* expression by Nutlin-activated p53. While activation of p53 by Nutlin-3 effectively reduced *Arf* expression by more than 50%

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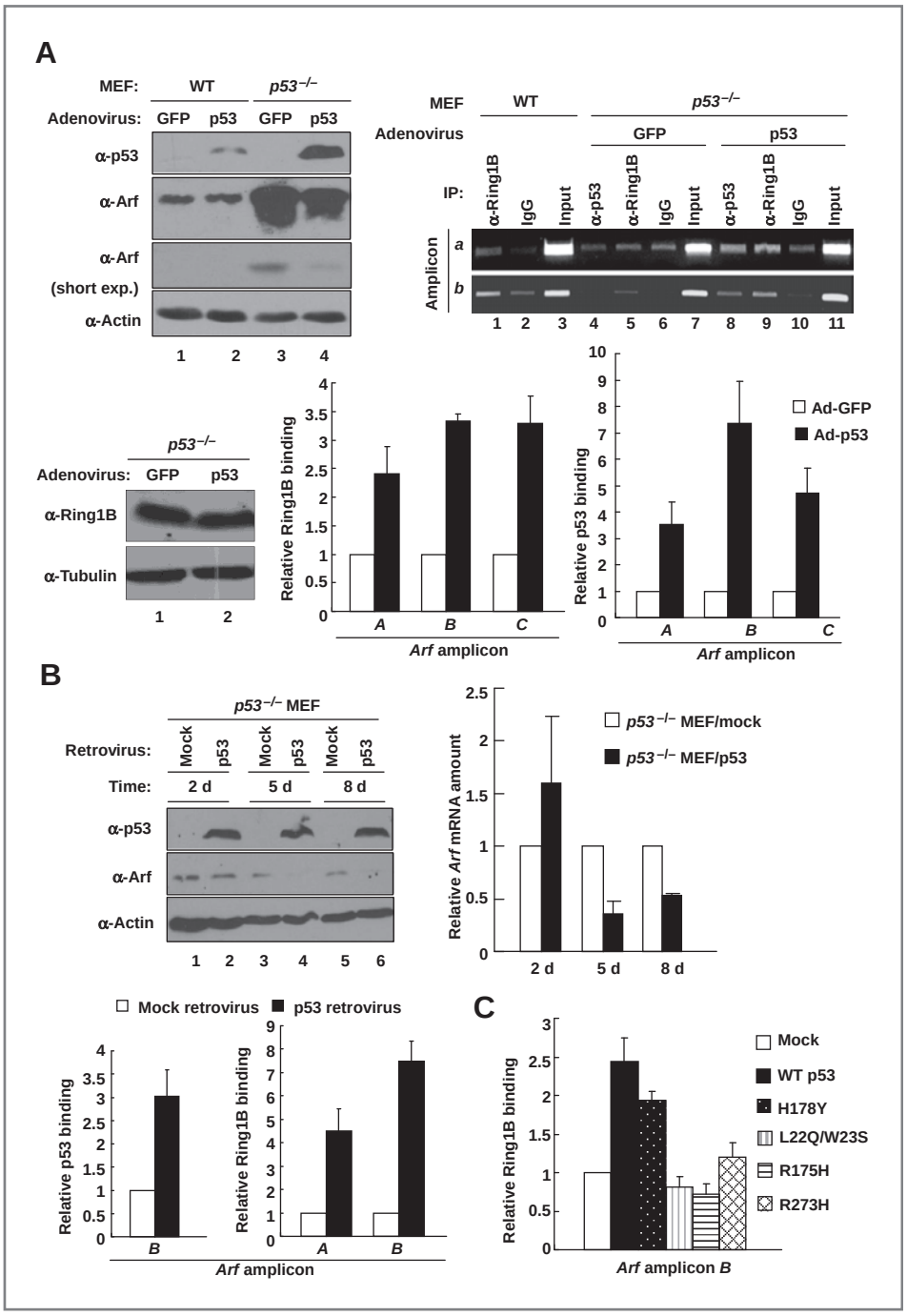

Figure 5. Both transactivity and DNA binding activity of p53 are required for PRC1 binding to *Arf*. A, WT (p3) and *p53*$^{-/-}$ MEFs were infected with either GFP- or p53-expressing adenovirus (Ad). Two days after infection, cell lysates were analyzed by WB (Western blot) for expression of p53 and Arf, and cross-linked cell pellets were collected and analyzed for binding of p53 and Ring1B to *Arf* locus. B, *p53*$^{-/-}$ MEFs were infected with either mock or p53-expressing retrovirus and selected by puromycin. The levels of p53 and Arf proteins or mRNA were determined by immunoblotting or Q-RT-PCR. Cells collected at 5 days after infection were analyzed for p53 and Ring1B binding on *Arf* by ChIP-Q-PCR. C, p53$^{-/-}$ MEFs were infected with either mock WT or mutant p53 retrovirus. Cells were selected by puromycin and analyzed for bindings of Ring1B to *Arf* by ChIP-Q-PCR using *Arf* amplicon *B*. GFP, green fluorescent protein.

in WT MEFs infected with shRNA control virus targeting luciferase, it had no significant effect to reduce *Arf* expression in MEFs transduced with any of the 3 shRNA virus targeting *Bmi1*. Hence, the function of p53 in the repression of *Arf* expression is also dependent on PcG.

Discussion

The results demonstrate a direct role of p53 in the repression of mouse *Arf* transcription by showing the direct binding of p53 to the mouse *Arf* locus and that p53 is required for recruiting HDAC and PcG proteins to the *Arf* locus. Our results are consistent with a model where p53 binds specifically to the *Arf* locus and then recruits HDAC to deacetylate the *Arf* locus. Deacetylation of the *Arf* locus then facilitates the recruitment of PRC to the *Arf* locus, leading to H3K27 trimethylation and silencing of *Arf* expression (Fig. 7C). This model is supported by 3 lines of evidence. First, *Arf* expression is elevated *in vivo* in multiple *p53*-deficient tissues or organs is rapidly elevated upon functional inactivation of p53 and is further repressed

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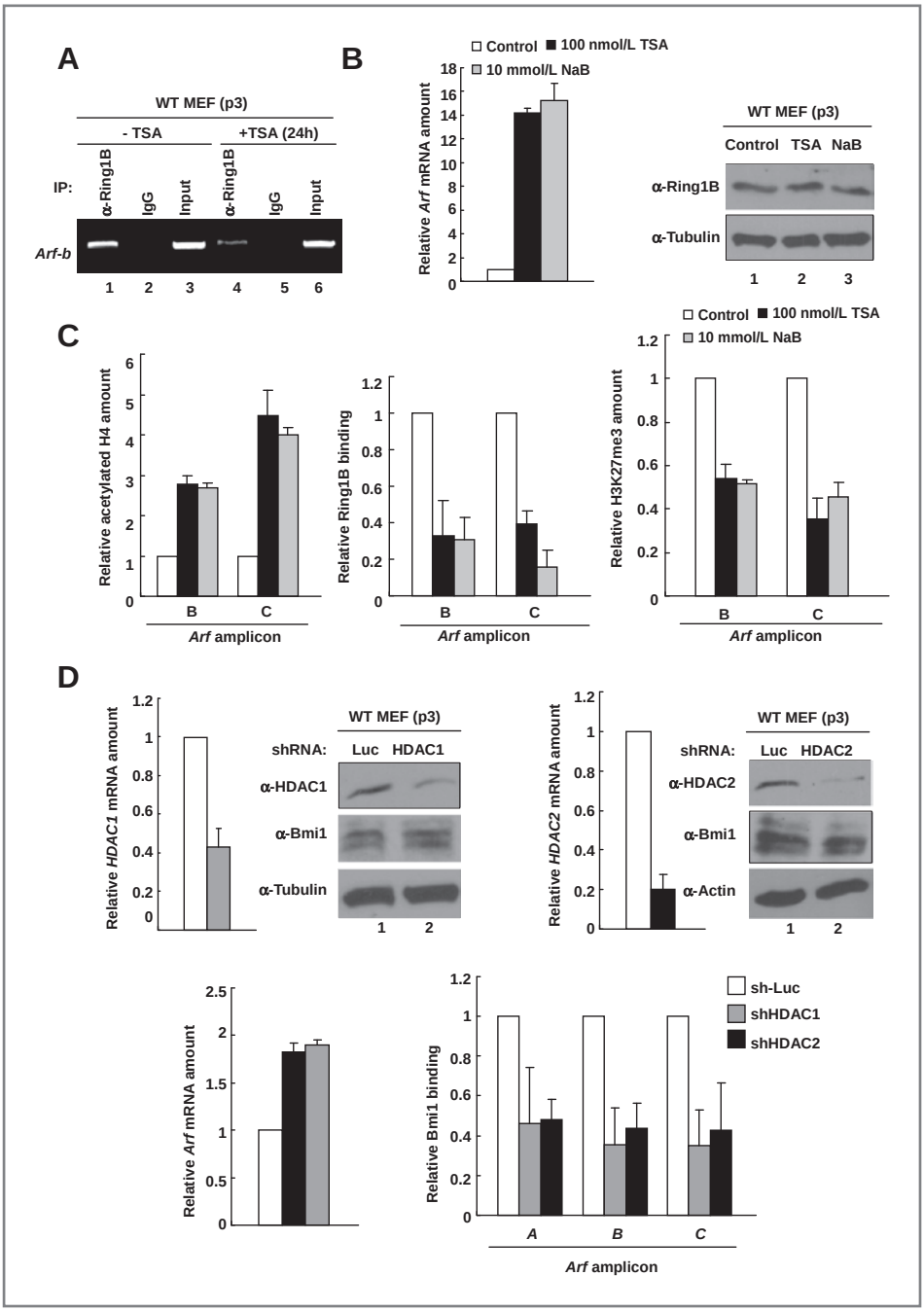

Figure 6. HDAC function is required for PRC to bind *Arf*. A, WT p3 MEFs were treated with 0.1 μmol/L TSA for 24 hours. Cell pellets were collected and analyzed for binding of Ring1B on *Arf* by ChIP. B, WT p3 MEFs were treated with 0.1 μmol/L TSA or 10 mmol/L NaB for 30 hours and cells were collected and analyzed for *Arf* mRNA amount by Q-RT-PCR. C, cells in B were collected and analyzed for histone H4 acetylation, histone H3K27 trimethylation, and Ring1B binding on *Arf* locus by ChIP-Q-PCR. D, WT MEFs were infected with either an sh-luciferase (Luc) or shHDAC-expressing retovirus, selected by puromycin for 2 days, and collected 4 days after infection. The mRNA levels of *HDAC1*, *HDAC2*, and *Arf* were determined by Q-RT-PCR, and the binding of Bmi1 to *Arf* locus was determined by ChIP-Q-PCR.

upon the activation of endogenous p53 in early passage of WT MEFs. Second, p53 directly binds to *Arf* locus, and that both transactivation and DNA binding activities of p53 are required for the repression of *Arf* and, importantly, for the binding to and repression of *Arf* by both HDAC and PRC. Third, we have also shown that both HDAC and PcG are conversely required for p53 to repress *Arf* expression.

The findings presented here shed mechanistic insights on the p53-mediated oncogenic checkpoint pathway: one on the mechanism of *Arf* activation and the other on the feedback regulation of p53. Although it has been observed for more than a decade that many hyperproliferative oncogenes can activate *Arf* expression (31), the molecular mechanism underlying oncogenic activation of *Arf* is unknown. Our demonstration that *Arf* is bound and repressed by p53 during normal cell growth suggests a critical step—dissociating p53 from the *Arf* locus—for an oncogene to activate *Arf* expression. It will be interesting to determine how an oncogene causes p53

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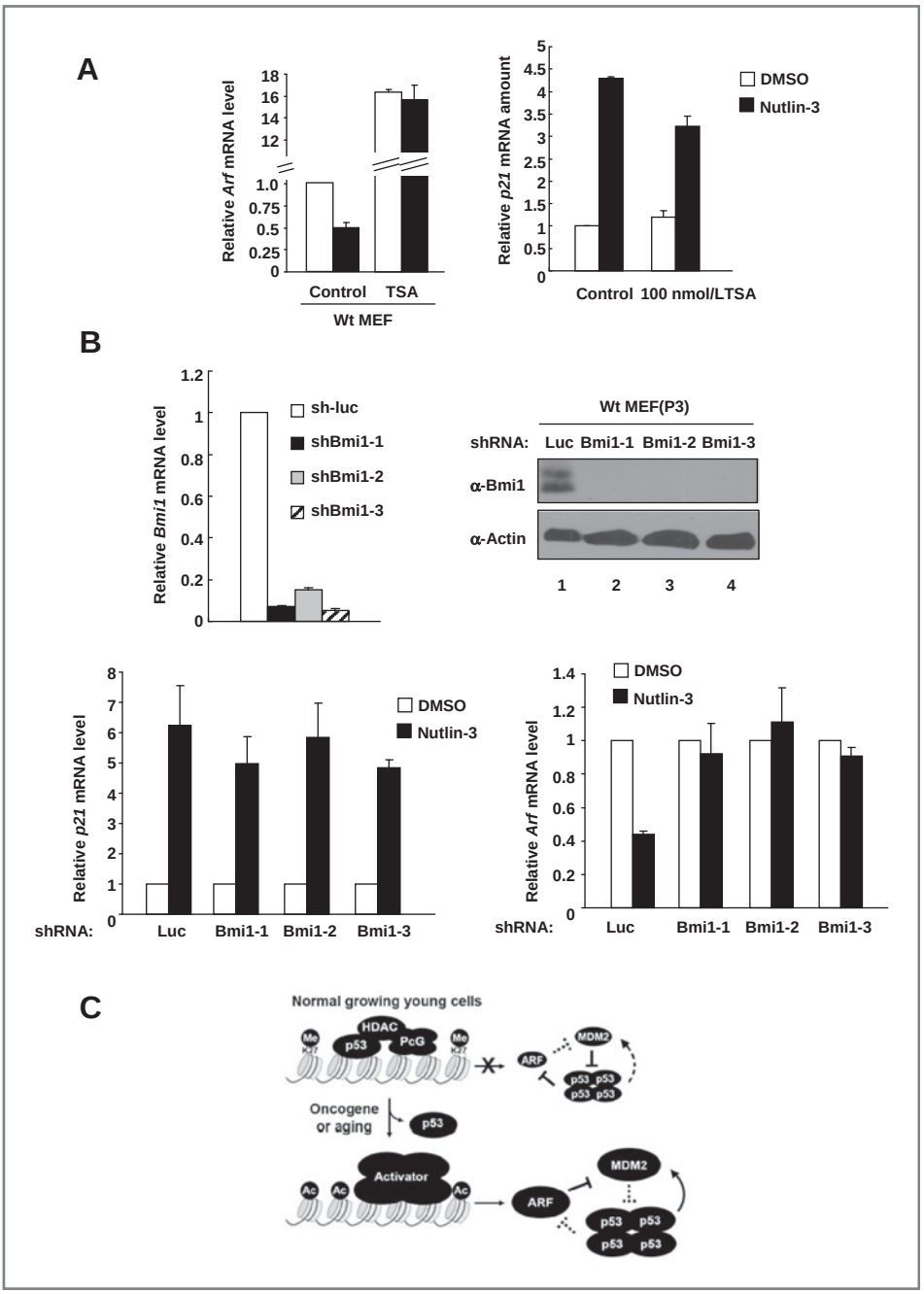

Figure 7. HDAC and PcG function are required for p53 to repress *Arf*. A, WT p2 MEFs were treated with TSA and Nutlin-3 either alone or in combination for 24 hours. Cells were collected and analyzed for mRNA levels of *p21* and *Arf* by Q-RT-PCR. B, WT MEFs (p2) were infected with either an sh-luciferase (Luc) or shBmi1-expressing retrovirus, selected by 2 μmol/L puromycin for 2 days, continuously cultured for 5 days after infection, and then treated with Nutlin-3 for 24 hours. Cells were collected and analyzed for *Bmi1*, *p21*, and *Arf* mRNA level by Q-RT-PCR. C, a schematic model illustrating p53-mediated Arf repression by HDAC and PcG. See text for more details.

dissociation in the presence of increased level of p53 since *Arf* activation would lead to p53 stabilization.

Feedback inhibition is a regulatory strategy commonly used in biochemical reactions such as the inhibition of threonine dehydrase by isoleucine (45). The accumulation of an end product inhibits the enzyme involved in its synthesis to avoid excessive accumulation and waste of resources. A similar strategy is also widely employed in cell regulations, especially those involved in cell growth and proliferation, to ensure a balanced homeostasis and cell physiology. Feedback inhibition is particularly needed for the control of the function of a gene, such as *p53*, whose activity, if not feedback inhibited, could lead to an irreversible consequence to the cell such as permanent cell-cycle arrest or cell death. At low, nonlethal levels of DNA damage, cell-cycle progression is delayed by the activation of p53 and then p21 to give cells time to repair the DNA and then resumed when the repair is completed. The resumption of cell-cycle progression is achieved through the p53–MDM2 feedback loop in which p53 activates the transcription of its primary inhibitor (6–8). *ARF* gene expression

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exhibits a strong inverse correlation with the functional status of p53 in both human (19, 32) and mouse cells (33, 34), suggesting a possible feedback repression of *ARF* expression by p53. Ours results provide a molecular basis supporting this feedback regulation. The significance of evolving this second feedback inhibition loop is that the first p53–MDM2 negative feedback loop would not be effective to inhibit p53 to resume the cell cycle if *ARF* expression is not repressed: the continuously synthesized ARF would bind to and prevent MDM2 from degrading p53 (Fig. 7C).

Our study also adds to the understanding of p53-mediated transcriptional repression, an area that is much less understood than p53-mediated transcriptional activation although an estimated 15% of genes containing a p53 response element can be repressed by p53 (see recent review in refs. 2, 46). p53 has been reported to directly bind with mSin3A, a transcriptional corepressor and a member of class I HDAC complexes and recruit HDACs to a specific promoter such as *Map4* or *Nanog* (47, 48). We also confirmed the association between p53 and HDAC by detecting p53-HDAC1 binding in WT MEFs (Supplementary Fig. SD). Our study provides 2 separate lines of evidence supporting a role of histone deacetylation in p53-mediated repression of *Arf*. First, we showed that treatment of MEFs with a low concentration of TSA drastically increased *Arf* mRNA (>15-fold) and this effect is seen as early as 6 hours (Fig. 3). Second, we demonstrate that HDAC1 directly binds to and deacetylates *Arf* in a p53-dependent manner. We further identify a new mechanism—recruiting PRC—for p53-mediated transcriptional repression. To the best of our knowledge, this represents the first evidence that p53-mediated repression involves PRC which contains histone-modifying activities known to function in silencing gene expression. Conversely, identification of a sequence-specific binding factor—p53—in the recruitment of PRC to a specific locus also helps to better understand how PcG is recrutied to their targets, a puzzling issue associated with the repression of many PRC-regulated genes in mammalian cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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