Bidirectional autoregulatory mechanism of metastasis-associated protein 1-alternative reading frame pathway in oncogenesis

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Although metastasis-associated protein 1 (MTA1), a component of the nucleosome remodeling and histone deacetylation complex, is widely up-regulated in human cancers and correlates with tumor metastasis, its regulatory mechanism and related signaling pathways remain unknown. Here, we report a previously unrecognized bidirectional autoregulatory loop between MTA1 and tumor suppressor alternative reading frame (ARF). MTA1 transactivates ARF transcription by recruiting the transcription factor c-Jun onto the ARF promoter in a p53-independent manner. ARF, in turn, negatively regulates MTA1 expression independently of p53 and c-Myc. In this context, ARF interacts with transcription factor specificity protein 1 (SP1) and promotes its proteasomal degradation by enhancing its interaction with proteasome subunit regulatory particle ATPase 6, thereby abrogating the ability of SP1 to stimulate MTA1 transcription. ARF also physically associates with MTA1 and affects its protein stability. Thus, MTA1-mediated activation of ARF and ARF-mediated functional inhibition of MTA1 represent a p53-independent bidirectional autoregulatory mechanism in which these two opposites act in concert to regulate cell homeostasis and oncogenesis, depending on the cellular context and the environment.

Chromatin-remodeling factors play a crucial role in the regulation of gene expression during cellular differentiation and development. One newly added group of ubiquitously expressed chromatin modifiers is the metastasis-associated protein (MTA) family, members of which play an integral role in nucleosome remodeling and histone deacetylation complexes that modify DNA accessibility to cofactors (1). MTA1, the founding member of the MTA family, functions not only as a transcriptional repressor of estrogen receptor-α (2) and breast cancer type 1 susceptibility protein (3) but also as a transcriptional activator of some genes such as breast carcinoma-amplified sequence 3 (BCAS3) (4) and paired box 5 (5), largely because of its ability to interact with RNA polymerase II (Pol II). MTA1 also is an essential downstream effector of c-Myc oncoprotein (6). Although MTA1 is widely up-regulated in human cancers and plays an important role in tumorigenesis and tumor metastasis (7), its regulatory mechanism and related signaling transduction pathways are not clear.

Through their ability to activate a number of progrowth and prosurvival pathways, oncogenes potently promote tumor initiation and progression (8). However, evolution has installed a variety of innate tumor-suppressive mechanisms in the proliferative program of mammalian cells that trigger apoptosis or senescence once proliferation becomes aberrant (9). One of the main mediators of the antioncogenic programs is the alternative reading frame (ARF) tumor suppressor (known as "p14ARF" in humans and "p19ARF" in mice; referred to as "ARF" hereafter), one of two products of the inhibitor of cyclin-dependent kinase 4a (INK4a)/ARF locus on chromosome 9p21 (10). The principal function of ARF is to counteract hyperproliferative signals emanating from constitutively activated oncogenes through modulating the activity of p53 transcription factor by antagonizing the p53-specific ubiquitin ligase mouse double minute 2 (Mdm2) (11). Recently, ARF has

been reported to associate with proteins other than Mdm2 and to have p53-independent tumor-suppressive activities (11). For example, mice null for ARF, p53, and Mdm2 experienced tumor development at a higher frequency than did mice lacking both p53 and Mdm2 or p53 alone (12). These studies indicate that the tumor-suppressive functions of ARF are not elicited entirely through the Mdm2/p53 pathway and that additional cell factors must be targeted by ARF. In support of this notion, several ARF target proteins have been reported over the years (11), but these p53-independent activities of ARF remain poorly understood. Here, we report a p53-independent autoregulatory feedback loop between MTA1 and ARF in oncogenesis.

Results and Discussion

MTA1 Is a Transcriptional Coactivator of the ARF Gene. The ability of oncogenes to engage tumor-suppressor pathways represents a key regulatory mechanism that can limit the outgrowth of incipient tumor cells (8). To explore whether MTA1 interjects into the ARF-mediated tumor-suppressive pathway, we first evaluated the effect of endogenous MTA1 on the expression levels of ARF protein using mouse embryonic fibroblasts (MEFs) derived from wild-type and MTA1-knockout (MTA1$^{-/-}$) mice (13). Surprisingly, knockout of MTA1 resulted in a dramatic decrease in the expression levels of p19ARF protein in MTA1$^{-/-}$ MEFs relative to wild-type controls (Fig. 1A). Consistently, a significant decrease in the expression of MTA1 protein was accompanied by a drastic down-regulation of p19ARF protein in MTA1 siRNA-transfected cells compared with control siRNA-treated cells (Fig. 1B). By contrast, introduction of MTA1 in HC11 murine mammary epithelial cells led to a significant increase in p19ARF protein expression (Fig. 1C). Because HC11 cells carry two mutant p53 alleles and lack a functional p53 gene (14), we speculated that the regulation of ARF by MTA1 is p53 independent. To test this notion, we next depleted the endogenous MTA1 in p53$^{-/-}$/Mdm2$^{-/-}$ MEFs (15) using specific siRNAs targeting MTA1 and observed a similar effect of MTA1 on p19ARF protein expression (Fig. 1D), suggesting that MTA1 regulates p19ARF expression in a p53-independent manner.

To address the mechanism of MTA1 regulation of p19ARF, we next tested whether MTA1 affects p19ARF transcription. Results showed that the mRNA levels of the p19ARF gene decreased significantly in MTA1$^{-/-}$ MEFs compared with wild-type controls (Fig. 1E Left). In contrast, p19ARF mRNA levels were elevated significantly in HC11 stable clone cells overexpressing MTA1 (HC11/MTA1) compared with controls expressing empty
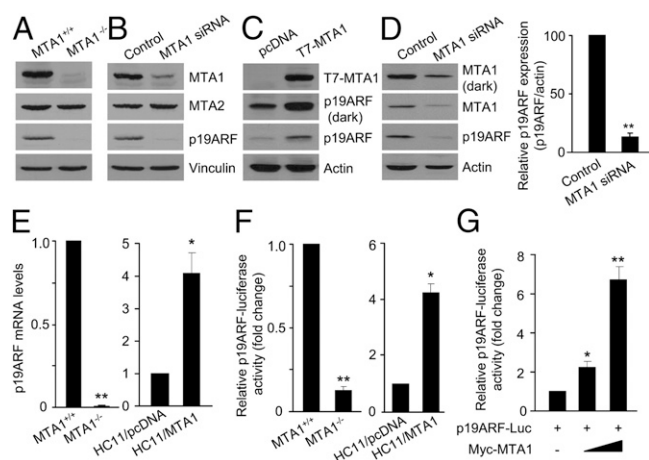
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Fig. 1. MTA1 regulates p19ARF expression. (A) Western blot analysis of the MTA1^{+/+} and MTA1^{-/-} MEFs with the indicated antibodies. Vinculin is shown as a loading control. (B) MTA1^{+/+} MEFs were transfected with specific siRNAs targeting MTA1 or control siRNAs and subjected to Western blot analysis with indicated antibodies. (C) Western blot analysis of HC11 cells stably expressing T7-MTA1 or empty vector (pcDNA) with the indicated antibodies. (D) (Left) p53^{-/-}/Mdm2^{-/-} cells were transfected with specific siRNAs targeting MTA1 or control siRNAs and were subjected to Western blot analysis with the indicated antibodies. The density of bands was measured using ImageQuant software version 7.0 (GE Healthcare) and normalized to that of β-actin. (Right) The fold change (p19ARF/β-actin) is shown. (E and F) Quantitative PCR analysis of ARF mRNA levels (E) or analysis of ARF promoter activity (F) in the MTA1^{+/+} and MTA1^{-/-} MEFs (Left) or the HC11/pcDNA and HC11/MTA1 cells (Right). (G) p53^{-/-}/Mmd2^{-/-} cells were transfected with the ARF-luciferase reporter plasmid (ARF-Luc) alone or in combination with increasing amounts of a Myc-MTA1 expression plasmid. ARF-luciferase activity was determined as described in *SI Appendix, SI Materials and Methods*. *P < 0.05; **P < 0.01.

vector (HC11/pcDNA) (Fig. 1E Right). These results suggest that MTA1 regulates p19ARF expression, at least in part, at the transcriptional level. In support of this notion, we found significantly reduced p19ARF promoter activity in MTA1^{-/-} MEFs relative to wild-type controls (Fig. 1F Left). In contrast, p19ARF promoter activity was elevated in HC11/MTA1 cells compared with HC11/pcDNA controls (Fig. 1F Right). Consistently, a dose-dependent increase in p19ARF promoter activity also was observed in p53^{-/-}/Mdm2^{-/-} cells in response to MTA1 expression (Fig. 1G). Collectively, these results clearly show that MTA1 functions as a coactivator of p19ARF transcription in a p53-independent manner.

To investigate whether the effect of MTA1 transactivation of ARF is limited to the murine system, we next validated these findings using human H1299 lung cancer cells, a p53-null cell line because of gene truncation (16) that has been used widely for investigating p53-independent cell function and signaling pathways. Human p14ARF is about 50% homologous to murine p19ARF, but mounting evidence suggests that the two proteins share a set of similar properties. For example, both p19ARF and p14ARF can interact directly with Mdm2 (11) and suppress cell adhesion and promote apoptosis (17). It is noteworthy that mouse MTA1 protein shares 94% homology with human MTA1 protein (18). We found that knockdown of endogenous MTA1 results in a significant down-regulation of p14ARF protein and mRNA expression in H1299 cells (*SI Appendix*, Fig. S1 *A and B*). We also observed a significant reduction in p14ARF promoter activity upon MTA1 knockdown (*SI Appendix*, Fig. S1C). These findings suggest that MTA1 transactivates both mouse and human ARF.

Recruitment of the c-Jun/MTA1/Pol II Coactivator Complex onto the ARF Promoter. To gain a deeper insight into the regulation of ARF transcription by MTA1, we next examined the recruitment of MTA1 onto the ARF promoter using a ChIP-based promoter walk analysis. We found that MTA1 was recruited onto five regions

(R1–R3, R5, and R6) of the p19ARF promoter in MTA1^{+/+} but not MTA1^{-/-} MEFs (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S2A). In support of this finding, a distinct increase in the recruitment of MTA1 onto these five regions of the ARF promoter was observed in HC11/MTA1 cells compared with HC11/pcDNA controls (Fig. 2C and *SI Appendix*, Fig. S2B).

Because MTA1 stimulated ARF transcription, we next tested the association of MTA1 and Pol II, an indicator of active transcription, on the ARF promoter. A single ChIP with an anti-RNA Pol II antibody revealed the recruitment of RNA Pol II to three regions (R1–R3) of p19ARF promoter (*SI Appendix*, Fig. S3). We also observed an enhanced recruitment of RNA Pol II onto these regions of the p19ARF promoter in HC11/MTA1 cells compared with HC11/pCDNA controls (*SI Appendix*, Fig. S3). We next carried out a double ChIP analysis using the HC11/pcDNA and HC11/MTA1 stable clone cells and found the MTA1/Pol II complex associates with two specific regions (R2 and R3) of the p19ARF promoter (Fig. 2 *A* and *D* and *SI Appendix*, Fig. S4).

Because MTA1 is a transcriptional coregulator that interacts with transcription factors either to activate or to repress the transcription of specific genes (1), we next performed a sequence analysis of the MTA1-ARF promoter-interacting regions using the Alibaba 2.1 program (http://www.gene-regulation.com/pub/programs/alibaba2/index.html?) to examine the nature of the putative transcription factor binding sites. Interestingly, we found two putative consensus binding sequences for c-Jun transcription factor in the MTA1-ARF promoter–interacting regions (*SI Appendix*, Fig. S5). We next tested the hypothesis that MTA1 is a coactivator of the ARF transcription, presumably through the recruitment of c-Jun transcription factor onto the ARF promoter. Indeed, the results of a double ChIP analysis demonstrated that MTA1 and c-Jun proteins were corecruited onto the p19ARF promoter (R2 and R3) (Fig. 2E and *SI Appendix*, Fig. S6). These findings were confirmed further by EMSA analysis. As shown in Fig. 2F, we found that the noted protein/ARF DNA complex could be supershifted by incubation of the nuclear extracts from the MTA1^{+/+} (Fig. 2F Left) but not MTA1^{-/-} (Fig. 2F Right) MEFs with a specific antibody against MTA1 (lane 3) or c-Jun (lane 4) but not control IgG (lane 6) at the expense of basal protein/ARF DNA complexes (lane 2), suggesting that both MTA1 and c-Jun proteins may interact with the ARF promoter. This notion was supported further by the finding that coincubation of anti-MTA1 and anti–c-Jun antibodies results in the formation of further higher-molecular-weight protein/DNA complexes (lane 5).

We next carried out a ChIP analysis to examine whether MTA1 and c-Jun also can recruit onto the human p14ARF promoter in H1299 cells. We found that MTA1 and c-Jun were recruited to the same region of the p14ARF promoter (R2) (*SI Appendix*, Fig. S7 *A and B*, respectively). Sequence analysis using the Alibaba 2.1 program revealed the conservation of the c-Jun consensus binding sequences in the human MTA1-p14ARF promoter–interacting region (*SI Appendix*, Fig. S7C). These results were validated further with a double ChIP wherein we observed that MTA1 followed by c-Jun also was recruited to the same region (R2) on the human p14ARF promoter (*SI Appendix*, Fig. S7D). These findings, along with the p14ARF promoter-luciferase studies (*SI Appendix*, Fig. S1C), suggest that MTA1 also transactivates human p14ARF expression by forming a coactivator complex along with c-Jun on its promoter.

Based on these findings, we next investigated the possibility of functional cooperation between MTA1 and c-Jun in the regulation of the ARF transcription. As expected, coexpression of MTA1 with c-Jun resulted in a cooperative activation of the ARF promoter activity in the p53^{-/-}/Mdm2^{-/-} MEFs (*SI Appendix*, Fig. S8A) and H1299 cells (*SI Appendix*, Fig. S8B). We next explored whether MTA1 could interact directly with c-Jun using GST pull-down assays. As shown in *SI Appendix*, Fig. S9A, ^{35}S-labeled, in vitro-translated c-Jun protein binds to the bromo-adjacent homology (amino acids 1–164) and C-terminal (amino acids 442–715) domains of MTA1, whereas MTA1 binds to the DNA-binding domain (DBD) (amino acids 257–281) of c-Jun protein (*SI Appendix*, Fig. S9B). Collectively, these results suggest that MTA1
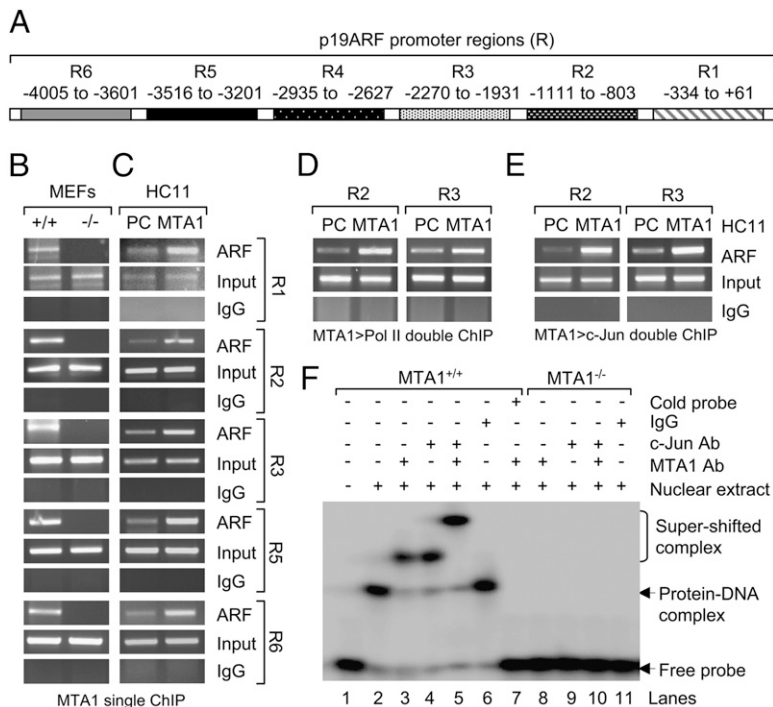
Fig. 2. Recruitment of the c-Jun/MTA1/Pol II coactivator complex onto the *ARF* promoter. (*A*) Line diagram showing the regions of *p19ARF* promoter analyzed. (*B* and *C*) ChIP analysis for the recruitment of MTA1 onto the *ARF* promoter in the MTA1$^{+/+}$ and MTA1$^{-/-}$ MEFs (*B*) or HC11/pcDNA and HC11/MTA1 cells (*C*). (*D*) Double ChIP analysis for the recruitment of the MTA1/Pol II complex onto the *ARF* promoter using HC11/pcDNA and HC11/MTA1 cells. The first ChIP with an anti-MTA1 antibody was followed by a second ChIP with an anti-Pol II antibody. (*E*) Double ChIP analysis for the recruitment of the MTA1/c-Jun complex onto the *ARF* promoter using HC11/pcDNA and HC11/MTA1 cells. The first ChIP with an anti-MTA1 antibody was followed by a second ChIP with an anti–c-Jun antibody. (*F*) EMSA analysis of MTA1/c-Jun protein complex binding to the *ARF* promoter using a PCR product encompassing the MTA1 and c-Jun consensus sequence in the MTA1$^{+/+}$ and MTA1$^{-/-}$ MEFs. Ab, antibody; PC, pcDNA.

stimulates *ARF* gene transcription by recruiting the c-Jun/MTA1/Pol II complex onto the *ARF* promoter.

ARF Negatively Regulates MTA1 Expression Independent of p53 and c-Myc. Because of the putative growth-inhibitory activity of ARF (11), we next determined the potential feedback effect of ARF on the MTA1 pathway. Interestingly, we observed a marked accumulation of MTA1, but not MTA2, protein in p19ARF$^{-/-}$ MEFs compared with wild-type controls (Fig. 3*A*). In contrast, induced expression of p19ARF inhibited the protein expression of MTA1 in p53$^{-/-}$/Mdm2$^{-/-}$ MEFs (Fig. 3*B*), indicating that the inhibitory effect of ARF on MTA1 expression is independent of the Mdm2/p53 pathway. Because ARF negatively regulates the expression of c-Myc oncoprotein (19), and MTA1 is the downstream effector of c-Myc (6), we next examined whether negative regulation of MTA1 by ARF is mediated by c-Myc. Results showed that induced expression of ARF resulted in markedly reduced levels of endogenous MTA1 protein in both c-Myc$^{+/+}$ and c-Myc$^{-/-}$ MEFs (20), indicating that the observed effect of ARF on MTA1 expression is also independent of c-Myc (Fig. 3*C*). We also observed that induced expression of ARF in c-Myc$^{+/+}$ MEFs resulted in a significant down-regulation of c-Myc expression (compare lane 2 and lane 1), and the protein levels of MTA1 in c-Myc$^{-/-}$ MEFs were reduced relative to c-Myc$^{+/+}$ controls (compare lane 3 and lane 1). These results enabled us to identify ARF as a bona fide negative regulator of MTA1 independent of p53 and c-Myc.

ARF Inhibits MTA1 Transcription. While investigating whether ARF affects the transcription of MTA1, we found that knockout of ARF resulted in a significant increase in the levels of MTA1 mRNA compared with wild-type controls (Fig. 3*D Left*). In contrast, induced expression of ARF in p53$^{-/-}$/Mdm2$^{-/-}$ cells resulted in a significant decrease in MTA1 mRNA levels (Fig. 3*D Right*). To validate the significance of these findings, we next analyzed the transcription levels of MTA1 and ARF in a published cDNA microarray database by Miller et al. (21) in which the global transcript profiles of 251 p53-sequenced primary breast tumors were analyzed using Affymetrix U133 oligonucleotide microarrays. We found an inverse correlation in the transcription levels of ARF and MTA1 genes in these breast tumors independent of p53 status (*SI Appendix*, Fig. S10). Furthermore, induced expression of ARF

in p53$^{-/-}$/Mdm2$^{-/-}$ cells inhibited *MTA1* promoter activity in a dose-dependent manner (Fig. 3*E*). Together, these results clearly suggest that ARF inhibits MTA1 expression, at least in part, at the transcriptional level independently of p53 and c-Myc.

ARF and SP1 Are Corecruited onto the *MTA1* Promoter. To understand the mechanism by which ARF inhibits MTA1 expression, we next used a ChIP-based promoter walk analysis to test whether ARF binds to the *MTA1* promoter and found that p19ARF was recruited to only one (R1) of the four regions of the *MTA1* pro-
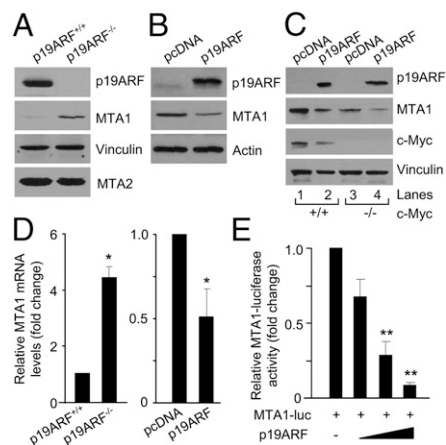

Fig. 3. ARF inhibits MTA1 expression independently of p53 and c-Myc. (*A*) Western blot analysis of the ARF$^{+/+}$ and ARF$^{-/-}$ MEFs with the indicated antibodies. (*B* and *C*) p53$^{-/-}$/Mdm2$^{-/-}$ (*B*) and c-Myc$^{+/+}$ and c-Myc$^{-/-}$ MEFs (*C*) were transfected with the indicated expression vectors and subjected to Western blot analysis with the indicated antibodies. (*D*) Quantitative PCR analysis of MTA1 mRNA levels in ARF$^{+/+}$ and ARF$^{-/-}$ MEFs (*Left*) and in p53$^{-/-}$/Mdm2$^{-/-}$ MEFs transfected with an ARF expression plasmid or empty vector control plasmid (*Right*). (*E*) p53$^{-/-}$/Mdm2$^{-/-}$ cells were transfected with a pGL3-MTA1–luciferase reporter plasmid alone or in combination with increasing amounts of an ARF expression plasmid, and MTA1-luciferase activity was determined as described above. *$P < 0.05$; **$P < 0.01$.

moter in the p19ARF$^{+/+}$ but not p19ARF$^{-/-}$ (negative control) MEFs (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S11). Sequence analysis of this region using the Alibaba 2.1 program revealed the presence of seven putative consensus binding sequences for the transcription factor specificity protein 1 (SP1) (*SI Appendix*, Fig. S12), indicating a possible contribution of SP1 to the transcriptional regulation of MTA1. ChIP analysis using an anti-SP1 antibody revealed that SP1 is recruited to three distinct regions (R1, R2, and R4) of the *MTA1* promoter in MTA1$^{+/+}$ but not MTA1$^{-/-}$ MEFs (Fig. 4*C* and *SI Appendix*, Fig. S13). We next determined whether SP1 protein binds to the *MTA1* promoter DNA using EMSA analysis of the nuclear extracts from ARF$^{-/-}$ MEFs. As shown in Fig. 4*D*, we found a distinct protein/MTA1 DNA complex that could be supershifted by incubation of the nuclear extracts with a specific antibody against SP1 (lane 3) but not ARF (negative control) (lane 4) or control IgG (lane 5) at the expense of basal protein/MTA1 DNA complexes (lane 2), confirming that SP1 protein interacts specifically with *MTA1* promoter DNA. In support of these findings, induced expression of SP1 dramatically stimulated *MTA1* transcription in a dose-dependent manner (*SI Appendix*, Fig. S14*A*, lanes 3–5). In contrast, treatment of cells with mithramycin A (MMA), an aureolic acid antibiotic that has been shown to inhibit SP1-mediated transcriptional activation selectively (22), resulted in a significant reduction in SP1-mediated transactivation of *MTA1* promoter activity (*SI Appendix*, Fig. S14*A*, lanes 6–8). Consistently, MMA-based treatment remarkably decreased the mRNA levels of MTA1 in a dose- and time-dependent manner (*SI Appendix*, Fig. S14*B*). These results suggest that SP1 is recruited onto the *MTA1* promoter and stimulates *MTA1* gene transcription.

To determine whether the ARF/SP1 complex associates with the murine *MTA1* promoter, we next performed a sequential double-ChIP analysis using the indicated antibodies and found that SP1 and ARF are corecruited only onto the ARF-*MTA1* promoter–interacting region R1 (Fig. 4*E* and *SI Appendix*, Fig. S15). To validate these findings further in human cells, we next carried out ChIP analyses using an anti-p14ARF antibody in the H1299 cells and found the recruitment of p14ARF onto two regions of the human *MTA1* promoter (R4 and R6) (*SI Appendix*, Fig. S16 *A* and *B*). A double ChIP analysis revealed the recruitment of both SP1 and p14ARF to only one region (R6) (*SI Appendix*, Fig. S16*C*). Sequence analysis using the Alibaba 2.1 program revealed the conservation of SP1 consensus binding sequences in this region (*SI Appendix*, Fig. S16*D*).

We next determined whether ARF/SP1 complex binds to *MTA1* promoter DNA using EMSA analysis. As shown in Fig. 4*F*, we found a distinct protein/MTA1 DNA complex that could be supershifted by incubation of the nuclear extracts from p19ARF$^{+/+}$ MEFs with a specific antibody against SP1 (lane 3) or ARF (lane 4) but not control IgG (lane 6) at the expense of basal protein/MTA1 DNA complexes (lane 2), suggesting that both ARF and SP1 proteins may interact with the *MTA1* promoter. This notion is supported by the finding that coincubation of anti-SP1 and anti-ARF antibodies resulted in the formation of further higher-molecular-weight protein/DNA complexes (lane 5).

ARF Promotes SP1 Proteasomal Degradation by Enhancing Its Interaction with Proteasome Subunit Regulatory Particle ATPase 6. Our findings that SP1 and ARF are corecruited onto the *MTA1* promoter raised the possibility of physical interaction between ARF and SP1 proteins. To test this notion, HEK293T cells were transfected with expression vectors encoding Flag-tagged SP1 and Myc-tagged ARF, and protein extracts were subjected to the sequential immunoprecipitation/Western blot analysis with the indicated antibodies. Results showed that exogenously expressed Flag-SP1 but not control IgG could be coimmunoprecipitated effectively with Myc-ARF, suggesting that the two proteins interact in vivo (Fig. 5 *A* and *B*). Furthermore, there was an interaction between SP1 and ARF at the endogenous protein level in p53$^{-/-}$/Mdm2$^{-/-}$ cells (Fig. 5*C*). Confocal scanning microscopy data also demonstrated that SP1 colocalizes with ARF in ARF$^{+/+}$ but not ARF$^{-/-}$ MEFs (Fig. 5*D*). To determine whether the interaction between
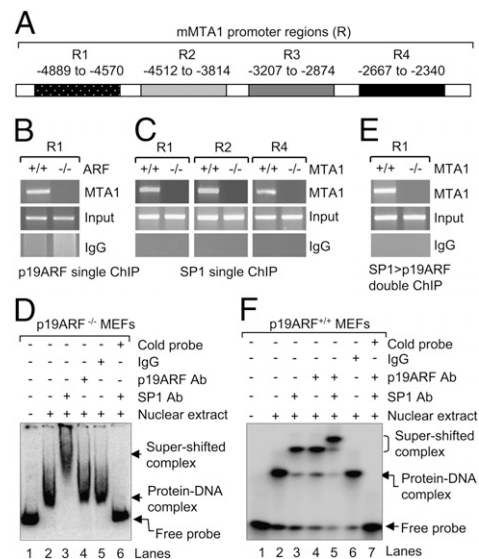

Fig. 4. ARF and SP1 are corecruited onto the *MTA1* promoter. (*A*) Line diagram showing the regions of the mMTA1 promoter analyzed. (*B* and *C*) ChIP analysis for the recruitment of ARF (*B*) or SP1 (*C*) onto the *MTA1* promoter as described above. (*D*) EMSA analysis of SP1 protein binding to the *MTA1* promoter DNA. (*E*) Double ChIP analysis for the recruitment of the SP1/ARF complex onto the *MTA1* promoter using MTA1$^{+/+}$ and MTA1$^{-/-}$ MEFs (negative controls). The first ChIP with an anti-SP1 antibody was followed by a second ChIP with an anti-p19ARF antibody. (*F*) EMSA analysis of SP1/ARF protein complex binding to the *MTA1* promoter DNA using nuclear extracts from p19ARF$^{+/+}$ MEFs.

ARF and SP1 is direct and to characterize their interacting domains, we performed GST pull-down assays. Results showed that ARF protein binds efficiently to the B (lane 5) and DBD domains of SP1 (lane 8) (*SI Appendix*, Fig. S17*A*), whereas SP1 binds to the N-terminal region of ARF protein (*SI Appendix*, Fig. S17*B*).

We next examined the possibility that ARF promotes degradation of SP1, thereby abrogating its coactivator activity upon *MTA1* transcription. Indeed, we found that the protein levels of SP1 were increased significantly in ARF$^{-/-}$ MEFs compared with wild-type controls (Fig. 5*E*). Furthermore, induced expression of ARF in p53$^{-/-}$/Mdm2$^{-/-}$ cells distinctly reduced the endogenous SP1 protein expression in a dose-dependent manner (Fig. 5*F*). Interestingly, the ARF-mediated degradation of SP1 was blocked effectively by treating cells with the proteasome inhibitor MG132 (Fig. 5*G*), suggesting that down-regulation of SP1 by ARF was occurring, at least in part, through the proteasome (although we could not rule out the possibility of other alternative mechanisms in the noted down-regulation of SP1 by ARF).

In line with these findings, we next observed that ARF distinctly potentiated the levels of SP1 ubiquitination and that this modification was accompanied by SP1 down-regulation (*SI Appendix*, Fig. S18*A*). Previous studies revealed that a subunit of the 26S proteasome, regulatory particle ATPase 6 (Rpt6), could interact with SP1 (23), and the interaction increases the efficiency of SP1 proteolytic processing and ubiquitination and then results in SP1 degradation (24). We next examined whether ARF augments the interaction of SP1 with Rpt6, resulting in increased proteasome degradation of SP1. As shown in *SI Appendix*, Fig. S18*B*, knockout of ARF decreased the interaction of SP1 with Rpt6 (compare lane 3 and lane 2) at endogenous levels. In contrast, increased accumulation of SP1 protein was shown clearly in ARF$^{-/-}$ cells compared with wild-type controls (compare lane 6 and lane 5). Thus, we concluded that ARF interacts with SP1 and promotes its proteasomal degradation by enhancing its interaction with proteasome subunit Rpt6, thereby abrogating the ability of SP1 to stimulate *MTA1* transcription. However, because the SP1-ARF complex is recruited to the *MTA1* promoter, we could not rule out the possibility that ARF localized to the *MTA1* promoter prevents
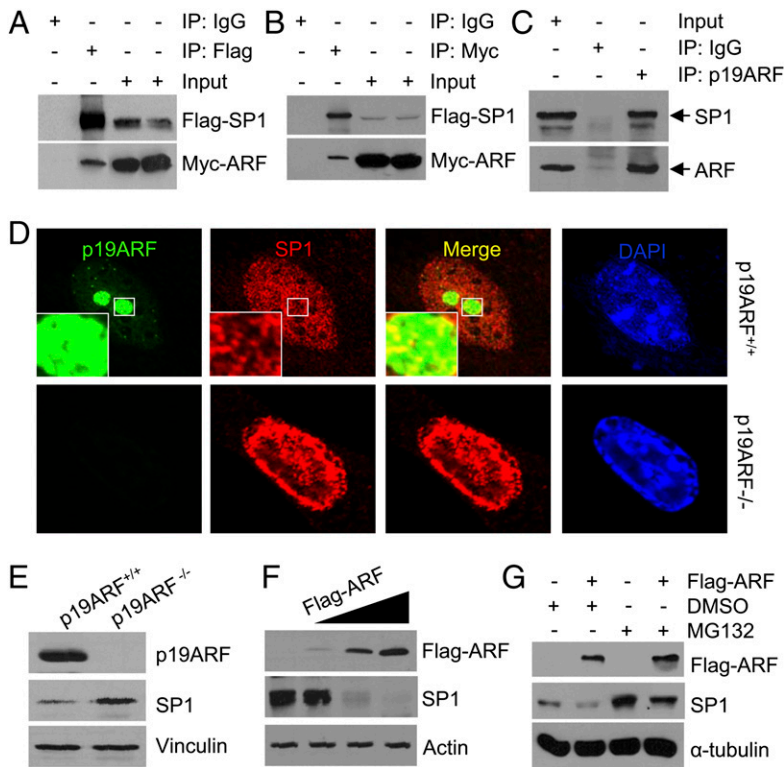
Fig. 5. ARF associates with SP1 and promotes its proteasome degradation. (*A* and *B*) HEK293T cells were transfected with expression vectors encoding Myc-ARF and Flag-SP1. Protein extracts were immunoprecipitated with control IgG or an anti-Flag antibody (*A*) or with anti-Myc antibody (*B*) and were immunoblotted with the indicated antibodies. (*C*) Protein extracts from p53$^{-/-}$/Mdm2$^{-/-}$ cells were subjected to immunoprecipitation analysis with an anti-p19ARF antibody or control IgG, followed by Western blotting using the indicated antibodies. (*D*) ARF$^{+/+}$ or ARF$^{-/-}$ MEFs were incubated with anti-SP1 and anti-p19ARF antibodies and then were labeled with Alexa Fluor 488- and Texas Red-conjugated secondary antibodies and analyzed by confocal microscopy. (*E*) Western blot analysis of the protein expression of SP1 and ARF in the ARF$^{+/+}$ and ARF$^{-/-}$ MEFs. (*F*) p53$^{-/-}$/Mdm2$^{-/-}$ cells were transfected with or without increasing amounts of a Flag-ARF expression plasmid and subjected to immunoblotting with the indicated antibodies. (*G*) p53$^{-/-}$/Mdm2$^{-/-}$ cells were transfected with a Flag-ARF expression vector or empty vector control plasmid. After 48 h of transfection, cells were treated with DMSO or 20 μM of MG132 for 6 h and then were immunoblotted with the indicated antibodies.

SP1-mediated transcription. We further demonstrated that more SP1 protein was recruited onto the *MTA1* promoter in ARF$^{-/-}$ MEFs than in ARF$^{+/+}$ controls (*SI Appendix*, Fig. S19) and that exogenous ARF inhibited both basal and SP1-mediated stimulation of *MTA1* promoter activity (*SI Appendix*, Fig. S20).

ARF Affects the Protein Stability of MTA1. Previous studies have demonstrated that ARF affects protein stability of its binding partners by inducing 26S proteasome-mediated degradation (11). Recently, MTA1 protein has been shown to be targeted for degradation by a ubiquitin–proteasome pathway (25). Therefore, we next tested whether ARF also affects the protein stability of MTA1 in addition to its characterized role in *MTA1* transcription. To this aim, we first tested whether ARF interacts with MTA1 in p53$^{-/-}$/Mdm2$^{-/-}$ cells transfected with an expression vector encoding Myc-tagged MTA1 (Myc-MTA1) alone or in combination with Flag-tagged ARF (Flag-ARF). As shown in *SI Appendix*, Fig. S21*A*, Myc-MTA1 and Flag-ARF are coimmunoprecipitated only when coexpressed (lane 2), indicating that these two proteins can interact in vivo and that the interaction of both proteins is specific because Flag-ARF is not immunoprecipitated when Myc-MTA1 is expressed alone (lane 1). More importantly, using MTA1$^{-/-}$ MEFs (13) as negative controls, we found an interaction between the endogenous MTA1 and ARF in MTA1$^{+/+}$ but not in MTA1$^{-/-}$ MEFs (*SI Appendix*, Fig. S21*B*), suggesting that ARF and MTA1 proteins can form a stable complex in physiologically relevant settings. It also was noticed that induced expression of ARF protein in p53$^{-/-}$/Mdm2$^{-/-}$ cells resulted in a significant down-regulation of exogenously expressed Myc-MTA1 (*SI Appendix*, Fig. S21*A*). These results indicate that ARF also could affect the MTA1 protein function, in addition to its role in *MTA1* transcription. In support of this notion, we found that ARF-mediated degradation of MTA1 in H1299 cells was partially blocked by treating the cells with the proteasome inhibitor MG132 (*SI Appendix*, Fig. S21*C*), suggesting that the ubiquitin–proteasome pathway also is involved in the regulation of MTA1 by ARF. Further, we observed that ARF distinctly potentiates the levels of MTA1 ubiquitination, and this modification is accompanied by MTA1 down-regulation (*SI Appendix*, Fig. S21*D*). Together, these results strongly support the no-

tion that ARF might affect the protein stability of MTA1 in addition to affecting *MTA1* transcription.

ARF Inhibits MTA1-Mediated Transactivation Activity and Cell Migration and Invasion. We next sought to determine whether ARF influences the biologic functions of MTA1. To this end, we first tested the effect of ARF on the activity of MTA1-responsive promoters. We found that induced expression of MTA1 alone increases *BCAS3* and *NF-κB* promoter activity in the HC11 cells, as reported previously (4, 26). However, coexpression of ARF counteracted the ability of MTA1 to transactivate the *BCAS3* (*SI Appendix*, Fig. S22*A*) and *NF-κB* promoters (*SI Appendix*, Fig. S22*B*).
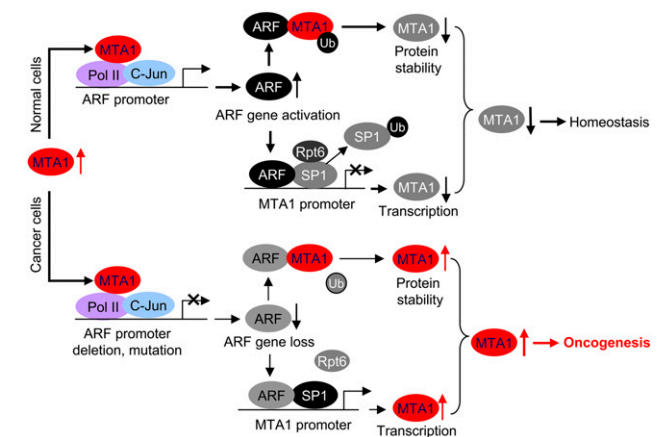

Fig. 6. An integrated working model. ARF is induced by MTA1-mediated oncogenic signals through the corecruitment of c-Jun onto the *ARF* promoter. ARF, in turn, inhibits MTA1 expression by blocking the SP1-mediated transactivation of MTA1 or affecting its protein stability. This inhibition creates a bidirectional autoregulatory feedback loop that acts in concert to regulate cell homeostasis and oncogenesis, depending on the cellular context and the environment.

MTA1 has been identified as one of the critical players in cancer cell migration and invasion (27), and emerging evidence has shown that ARF negatively regulates cell migration and invasion by targeting different signaling pathways (28). In light of these findings, we next determined whether ARF counteracts the MTA1-mediated migratory and invasive potential of cancer cells. Boyden chamber assay revealed that coexpression of ARF attenuates MTA1-mediated cell migration and invasion (*SI Appendix*, Fig. S22C). These results suggest that the observed bidirectional regulatory relationship between ARF and MTA1 has functional implications in counteracting the oncogenic activity of MTA1.

To characterize further the significance of these findings, we analyzed the correlation of ARF-high/MTA1-low and ARF-low/MTA1-high transcriptional levels with disease-specific survival in 251 primary breast tumors (21). When wild-type p53 was present, the patterns of the MTA1/ARF transcriptional levels had no significant effect on disease-specific survival of these patients with primary breast tumors (*SI Appendix*, Fig. S23 *A* and *B*). Interestingly, ARF-low/MTA1-high transcriptional levels correlated well with reduced disease-specific survival in breast cancer patients harboring mutant p53 (*SI Appendix*, Fig. S23C). Although it is not surprising that the disease-specific survival in human breast cancer patients was reduced with the lack of the tumor suppressors p53 and ARF (*SI Appendix*, Fig. S23C, red line), the distinct effect of ARF-high/MTA1-low transcriptional levels on the disease-specific survival is evidently shown (*SI Appendix*, Fig. S23C, green line) in the absence of p53, emphasizing the possible major p53-independent role of ARF. These findings further highlight the primary importance of p53 functional status in predicting the behavior of breast cancer (23). As a result, the functional role of the MTA1–ARF loop may be overlapped in breast tumors expressing wild-type p53. In the absence of functional p53, however, the MTA1–ARF regulatory loop would exert its dominant role in breast cancer prognosis.

In summary, the findings presented here establish that the *ARF* gene is induced by MTA1-mediated oncogenic signals through the corecruitment of c-Jun onto the *ARF* promoter. ARF, in turn, inhibits MTA1 expression by blocking transcription factor SP1-mediated transactivation or by affecting its protein stability, thus creating an autoregulatory feedback loop that regulates both MTA1 and ARF activities in a p53-independent manner (Fig. 6). Given the evidence that MTA1 transactivates ARF and ARF counteracts MTA1, the interplay between the ARF tumor suppressor and the MTA1 oncogene is likely to maintain a delicate control of cell homeostasis in normal cells. When the amount of MTA1 exceeds normal cellular proliferation levels, a safeguard mechanism needs to be activated whereby MTA1 induces ARF, which in turn down-regulates MTA1 (Fig. 6 *Upper*). As is often the case with p53, the *ARF/INK4A* locus is inactivated by complete deletion or mutation or aberrant promoter methylation in about 30% of all known types of malignancies (29). Thus, the loss of ARF in cancer cells could contribute to MTA1-induced tumorigenesis by at least two mechanisms. The down-regulation of ARF results in unrestricted MTA1-mediated cell proliferation and transformation resulting from the absence of an "oncogenic checkpoint." Another important consequence of ARF suppression or loss might result in increased MTA1 levels and signaling through a negative feedback mechanism, further fueling the oncogenic activity of MTA1 (Fig. 6 *Lower*). Therefore, bidirectional ARF–MTA1 signaling represents an important fail-safe mechanism for preventing MTA1-mediated tumorigenesis and tumor progression through the control of MTA1 activities. Certainly, further investigations of the ARF–MTA1 axis in mouse and human cancer are likely to yield important insights into mechanisms of tumor progression, providing leads for the therapeutic targeting of this pathway in cancer biology.

Materials and Methods

HEK293T and H1299 cells were obtained from ATCC. p53$^{-/-}$/Mdm2$^{-/-}$, p19ARF$^{-/-}$, and c-Myc$^{-/-}$ MEFs and their corresponding wild-type counterparts were kindly provided by Guillermina Lozano (M.D. Anderson Cancer Center, Houston, TX), Yanping Zhang (University of North Carolina at Chapel Hill, Chapel Hill, NC), and Ignacio Moreno de Alborán (National Center for Biotechnology, Madrid, Spain), respectively. MTA1$^{+/+}$ and MTA1$^{-/-}$ MEFs were generated in our laboratory from embryos at day 9 of development (13). All cell lines were grown in media recommended by the providers supplemented with 10% FBS and 1× antibiotic-antimycotic solution in a humidified 5% CO_2 atmosphere at 37 °C. Materials and methods used are discussed in detail in *SI Appendix, SI Materials and Methods*. Primers used for ChIP assay are provided in *SI Appendix*, Tables S2–S5, and primers used for quantitative PCR analysis are given in *SI Appendix*, Table S6.

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