ARF-BP1/Mule Is a Critical Mediator of the ARF Tumor Suppressor

Delin Chen,[1] Ning Kon,[1] Muyang Li,[1]
Wenzhu Zhang,[1] Jun Qin,[2] and Wei Gu*
[1]Institute for Cancer Genetics
Department of Pathology
College of Physicians and Surgeons
Columbia University
1150 St. Nicholas Avenue
New York, New York 10032
[2]Departments of Biochemistry and Cell Biology
Baylor College of Medicine
One Baylor Plaza
Houston, Texas 77030

Summary

Although the importance of the ARF tumor suppressor in p53 regulation is well established, numerous studies indicate that ARF also suppresses cell growth in a p53/Mdm2-independent manner. To understand the mechanism of ARF-mediated tumor suppression, we identified a ubiquitin ligase, ARF-BP1, as a key factor associated with ARF in vivo. ARF-BP1 harbors a signature HECT motif, and its ubiquitin ligase activity is inhibited by ARF. Notably, inactivation of ARF-BP1, but not Mdm2, suppresses the growth of p53 null cells in a manner reminiscent of ARF induction. Surprisingly, in p53 wild-type cells, ARF-BP1 directly binds and ubiquitinates p53, and inactivation of endogenous ARF-BP1 is crucial for ARF-mediated p53 stabilization. Thus, our study modifies the current view of ARF-mediated p53 activation and reveals that ARF-BP1 is a critical mediator of both the p53-independent and p53-dependent tumor suppressor functions of ARF. As such, ARF-BP1 may serve as a potential target for therapeutic intervention in tumors regardless of p53 status.

Introduction

The p53 protein has been described as a "guardian of the genome" because of its crucial role in coordinating cellular responses to stress (Lane, 1992; Levine, 1997). The antiproliferative effects of p53 are imparted through a variety of mechanisms that include cell cycle arrest, apoptosis, and cellular senescence/aging (Vogelstein et al., 2000; Lowe and Sherr, 2003). p53 can be thought of as the central node of a regulatory circuit that monitors signaling pathways from diverse sources, including DNA damage responses (e.g., ATM/ATR activation), abnormal oncogenic events (e.g., Myc or Ras activation), and everyday cellular processes (e.g., growth factor stimulation) (Giaccia and Kastan, 1998; Prives and Hall, 1999; Vousden and Lu, 2002; Brooks and Gu, 2003). While p53 mutations have been documented in more than half of all human tumors (Hollstein et al., 1999), defects in other components of the p53 pathway, such

as the ARF tumor suppressor, are observed in tumor cells that retain wild-type p53 (Sherr, 2001; Sharpless and DePinho, 2004). Thus, inactivation of the p53 pathway appears to be a common, if not universal, feature of human cancer.

The cellular functions of p53 are rapidly activated in response to stress. Although the mechanisms of p53 activation are not fully understood, they are generally thought to entail posttranslational modifications of p53, mainly including ubiquitination, phosphorylation, and acetylation (Brooks and Gu, 2003; Giaccia and Kastan, 1998). Ubiquitination of p53 was first discovered in papillomavirus-infected cells, where p53 degradation is mediated by the viral E6 protein and a HECT domain-containing ubiquitin ligase called E6-AP (Munger and Howley, 2002). In normal cells, Mdm2, a RING finger oncoprotein, acts as a specific E3 ubiquitin ligase for p53 (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997; Fuchs et al., 1998; Fang et al., 2000), which, if malignantly activated, has the potential to counteract the tumor suppressor functions of p53 (Michael and Oren, 2003). The critical role of Mdm2 in regulating p53 is best illustrated by studies carried out in mice where inactivation of p53 was shown to completely rescue the embryonic lethality caused by loss of Mdm2 function (Jones et al., 1995; Montes de Oca Luna et al., 1995).

Although earlier studies suggested that Mdm2 is the primary factor controlling p53 turnover, there is growing evidence that p53 degradation is more complex than originally anticipated. We recently found that Mdm2 differentially catalyzes either monoubiquitination or polyubiquitination of p53 in a dosage-dependent manner (Li et al., 2003). It seems likely that these distinct mechanisms are exploited in different physiological settings. For example, Mdm2-mediated polyubiquitination and nuclear degradation may play a critical role in suppressing p53 function during the latter stages of a DNA damage response or when Mdm2 is malignantly over-expressed (Xirodimas et al., 2001a; Shirangi et al., 2002). On the other hand, Mdm2-mediated monoubiquitination and subsequent cytoplasmic translocation of p53 may represent an important means of p53 regulation in unstressed cells, where Mdm2 is maintained at low levels (Freedman et al., 1999; Stommel et al., 1999; Boyd et al., 2000; Geyer et al., 2000). Moreover, deubiquitination of either p53 or Mdm2 by HAUSP is apparently a critical event in these dynamic processes (Li et al., 2004; Cummins et al., 2004), and additional cellular factors are necessary to facilitate p53 degradation in normal cells. Indeed, it was recently reported that the ubiquitin ligases COP1 and Pirh2 are directly involved in p53 degradation (Leng et al., 2003; Dornan et al., 2004). Taken together, these studies suggest that, while Mdm2 is a key regulator of p53 function, p53 degradation is mediated through both Mdm2-dependent and Mdm2-independent pathways in vivo.

ARF (known as p14[ARF] in humans and p19[ARF] in mouse) was originally identified as an alternative transcript of the Ink4a/ARF tumor suppressor locus, a gene that encodes the p16[Ink4a] inhibitor of cyclin-dependent

*Correspondence: wg8@columbia.edu

kinases (Sherr, 2001). By virtue of its unique first exon, the ARF transcript encodes a protein that is unrelated to p16[Ink4a] (Quelle et al., 1995). Nevertheless, ARF, like p16[Ink4a], exhibits tumor suppressor functions, as demonstrated by the tumor susceptibility phenotype of p14[ARF]-deficient mice (Kamijo et al., 1997). ARF suppresses aberrant cell growth in response to oncogene activation, at least in part, by inducing the p53 pathway (Sherr, 2001; Sharpless and DePinho, 2004). The ARF induction of p53 appears to be mediated through Mdm2, since overexpressed ARF interacts directly with Mdm2 and inhibits its ability to promote p53 degradation (Pomerantz et al., 1998; Zhang et al., 1998; Kamijo et al., 1998). Interestingly, the mechanisms by which ARF modulates the Mdm2/p53 pathway appear to be complex. On one hand, ARF is predominately a nucleolar protein that can stabilize nucleoplasmic p53 by binding and sequestering Mdm2 in the nucleolus (Weber et al., 1999). On the other hand, nucleoplasmic forms of ARF also appear to activate p53 function (Llanos et al., 2001; Lin and Lowe, 2001) by directly inhibiting the ubiquitin ligase activity of Mdm2 (Honda and Yasuda, 1999; Midgley et al., 2000).

Notably, ARF also has tumor suppressor functions that do not depend on p53 or Mdm2. For example, ARF can induce cell growth arrest in tumor cells that lack a functional *p53* gene (Normand et al., 2005; Yarbrough et al., 2002; Eymin et al., 2003; Weber et al., 2000; Rocha et al., 2003; Korgaonkar et al., 2002) or a gene encoding the p21 cyclin-dependent kinase inhibitor, a key transcriptional target of p53 (Modestou et al., 2001). ARF can also suppress the proliferation of MEFs lacking both *Mdm2* and *p53* (Weber et al., 2000; Kuo et al., 2003). Consistent with these findings, the tumor susceptibility of triple knockout mice that lack *Arf*, *p53*, and *Mdm2* is significantly greater than that associated with mice lacking any one of these genes alone (Weber et al., 2000). Moreover, it was recently shown that ARF suppresses the growth, progression, and metastasis of mouse skin carcinomas through both p53-dependent and p53-independent pathways (Kelly-Spratt et al., 2004). Together, these studies imply the existence of distinct downstream factors that mediate the p53-independent functions of ARF. Indeed, this notion is also supported by the fact that p53 is not required for the developmental eye abnormalities with failed hyaloid vascular system displayed by *ARF*^{−/−} mice (McKeller et al., 2002). Nevertheless, the identity of these factors and the mechanisms by which they mediate p53-independent tumor suppression by ARF are not well understood.

To elucidate the p53/Mdm2-independent functions of ARF, we purified naturally formed ARF-containing protein complexes from p53 null human cells and identified ARF-BP1, a HECT (homology to E6-AP C terminus)-containing ubiquitin ligase, as a major component of these complexes. ARF-BP1 directly binds ARF, and its ubiquitin ligase activities are strongly inhibited by ARF. Significantly, inactivation of ARF-BP1 induces cell growth arrest in p53 null cells, indicating that ARF-BP1 is a critical mediator of the p53-independent pathway of ARF tumor suppression. Surprisingly, inactivation of ARF-BP1 in p53-positive cells induces p53 stabilization and activates a p53-dependent apoptotic response, implying that, in addition to Mdm2, ARF-BP1 also acts as a key factor in ARF-mediated p53 activation. These findings identify a novel regulatory pathway that mediates both the p53-independent and p53-dependent tumor suppressor functions of ARF.

Results

Identification of ARF-BP1 as a Major Component of ARF-Associated Nuclear Complexes from p53 Null Cells

To identify the in vivo targets for ARF-mediated functions, we used an epitope-tagging strategy to isolate ARF-containing protein complexes from human cells. Although we have used this approach previously (Gu et al., 1999; Luo et al., 2000; Nikolaev et al., 2003), several modifications of the method were introduced to improve the stoichiometry of the protein complexes. In particular, we generated a derivative of the human lung carcinoma p53 null H1299 cell line that stably expresses a double-tagged human ARF protein containing N-terminal HA and C-terminal Flag epitopes (HA-ARF-Flag) (Figure 1A). Furthermore, to avoid nonphysiological interactions that might occur in cells that overexpress ARF, we opted to use H1299 derivatives that express the ectopic ARF protein at levels similar to those of endogenous ARF (Figure 1B). Thus, the composition and stoichiometry of the tagged protein complexes are likely to reflect those of native ARF complexes.

To isolate protein complexes containing ARF, nuclear extracts from the stable cell lines were sequentially subjected to affinity chromatography on M2 (Flag antibody) agarose beads and an HA-affinity column. Finally, the bound proteins were fractionated by SDS-PAGE and visualized by silver staining (Figure 1C). As expected, we identified B23/nucleophosmin (NPM), a known ARF binding protein (Itahana et al., 2003; Bertwistle et al., 2004; Brady et al., 2004; Korgaonkar et al., 2005), as a specific component of the ARF complexes (Figure 1C). In addition, a major protein band of ∼500 kDa also copurified with ARF from HA-ARF-Flag-expressing H1299 cells (lane 2) but not from parental H1299 cells (lane 1), suggesting that this protein is a specific binding partner of ARF. Thus, we designated this protein as ARF-BP1 (ARF binding protein 1). Interestingly, however, significant levels of Mdm2 were not detected in these complexes by Western blot analysis (data not shown), and mass spectrometric analysis of additional minor bands that copurified with ARF (Figure 1C) failed to identify Mdm2 sequences. Thus, these data suggest that ARF-BP1 is a major component of the ARF-associated complexes in these cells.

Initial Characterization of the Ubiquitin E3 Ligase ARF-BP1

Peptide sequencing of the ARF-BP1 band by mass spectrometry identified two peptide sequences that matched a single, partial cDNA clone in the GenBank database. A small fragment of this protein (named UREB1) was previously reported to bind the preprodynorphin gene promoter, but its biological functions were not understood (Gu et al., 1994). Therefore, by rapid amplification of cDNA ends (RACE) and homology alignment with the partial cDNA sequences in the database, we assembled a full-length cDNA for human ARF-
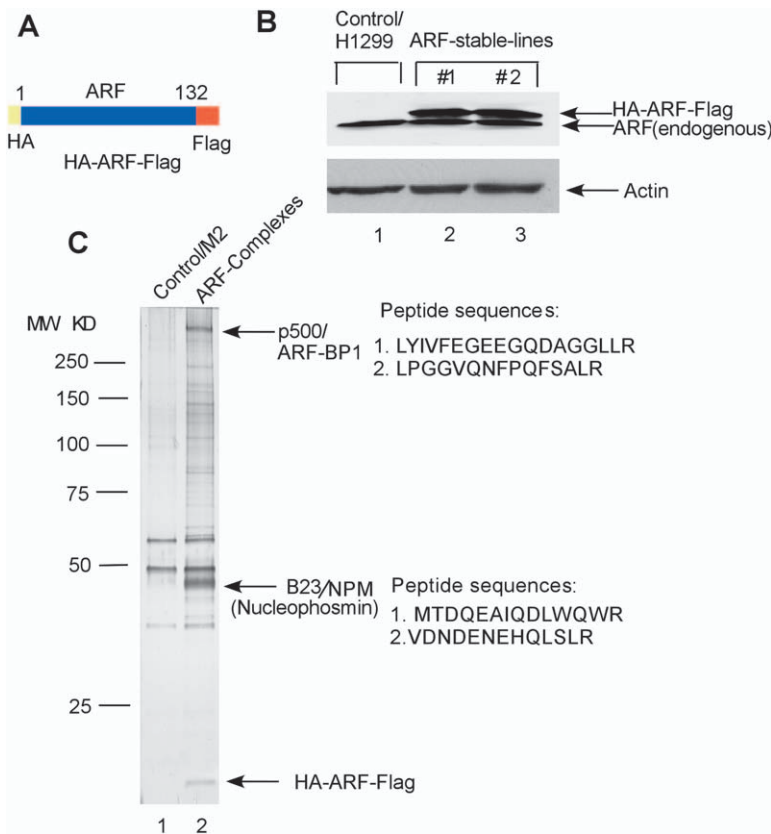
Figure 1. Identification of ARF-BP1 as a Major Component of the ARF-Associated Nuclear Complexes in Human Cells

(A) Schematic representation of the HA-ARF-Flag protein.

(B) The expression levels of HA-ARF-Flag and endogenous ARF in ARF stable lines. Western blot analysis of cell extracts from parental H1299 cell line (lane 1), ARF stable cell line clone 1 (lane 2), and ARF stable cell line clone 2 (lane 3) with an anti-ARF antibody.

(C) Silver staining of affinity-purified ARF complexes from a nuclear extract of the HA-ARF-Flag/H1299 stable cell line (lane 2) and a control elute from a parental H1299 nuclear cell extract (lane 1). Specific ARF-interacting protein bands were analyzed by mass spectrometry, and the p500/ARF-BP1 and B23/NPM (Nucleophosmin) peptide sequences are presented.

BP1 cDNA (accession number AY772009). This cDNA encodes a protein of 4374 amino acids (Figure 2A and Figure S1 in the Supplemental Data available with this article online), which is more than 3000 residues longer than the published UREB1 sequence (Gu et al., 1994).

The C-terminal sequences of ARF-BP1 possess a signature motif (the HECT domain) common to a number of ubiquitin E3 ligases (Figures 2A and 2B). This motif was first identified in E6-AP, a cellular E3 ligase that, when bound to the E6 protein of oncogenic human papillomaviruses, acquires the ability to bind and ubiquitinate the p53 tumor suppressor (Scheffner et al., 1993; Munger and Howley, 2002). The HECT domain, which encompasses about 350 amino acids, harbors a cysteine residue that forms a catalytic thiol ester with Ub and is essential for the enzymatic activity of HECT proteins (Pickart, 2001; Huang et al., 1999). ARF-BP1 also contains the ubiquitin-associated domain (UBA) (Figure 2A and Figure S2), a small sequence motif found in various proteins linked to the ubiquitination pathway, such as the DNA repair protein Rad23 or the Cbl ubiquitin ligase (Hicke and Dunn, 2003; Buchberger, 2002). A WWE domain was also identified, which may be involved in protein-protein interactions (Aravind, 2001). Northern blot analysis showed that the ARF-BP1 mRNA is broadly expressed in various types of human tissue (Figure 2C).

ARF-BP1 Interacts with ARF Both In Vitro and In Vivo

To confirm the physical interaction between ARF and ARF-BP1, we first tested whether ARF-BP1 binds ARF in vitro. The ARF polypeptide can be roughly divided into two major functional domains: an N-terminal region encoded by the unique 1β exon (N-ARF; residues 1–64), which is critical for ARF-mediated p53 activation as well as p53-independent ARF functions, and a C-terminal region (C-ARF; residues 65–132), which is not conserved between human and mouse counterparts and is of uncertain function. As shown in Figure 3A, ^{35}S-labeled ARF-BP1 (1–1014), a polypeptide comprising the N-terminal 1014 residues of ARF-BP1, did not associate with immobilized GST-ARF (lanes 7–9, Figure 3A). In contrast, however, ^{35}S-labeled ARF-BP1 (1015–4574) strongly bound both full-length ARF (GST-ARF; lane 3) and the N-terminal ARF domain (GST-N-ARF; lane 5) but not the C-terminal ARF domain (GST-C-ARF; lane 6) or GST alone (lane 2). Interestingly, ARF-BP1 weakly bound the ARF mutant GST-ARFΔ1–14 (lane 4), indicating that deletion of the N-terminal 14 amino acids of ARF significantly compromises but does not eliminate the ARF/ARF-BP1 interaction.

To confirm the interaction between ARF and ARF-BP1 in vivo, we raised an affinity-purified polyclonal antiserum against 192 amino acid segment of ARF-BP1 (residues 3435–3626) that shows no apparent homology with other known proteins. Upon Western blot analysis, this antibody specifically detects ARF-BP1 polypeptides in human cells (lane 1, Figure 3B). To investigate the interaction between endogenous ARF-BP1 and ARF polypeptides, cell extracts from native H1299 cells were immunoprecipitated with α-ARF-BP1 or with the control IgG. As expected, Western blot analysis revealed that this antibody immunoprecipi-
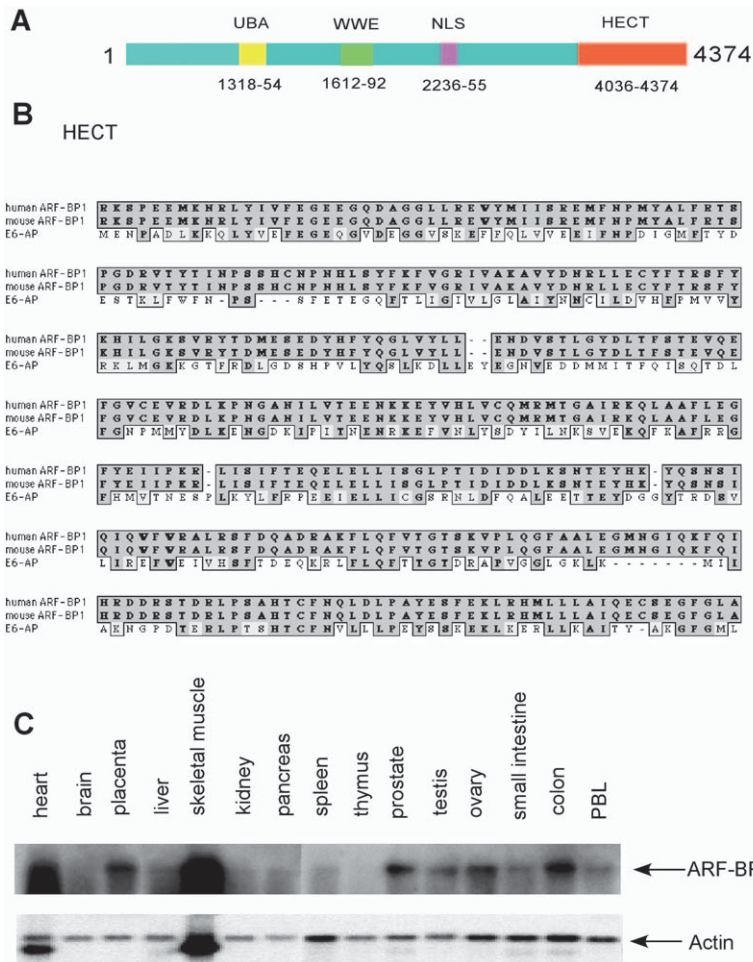
Figure 2. ARF-BP1 Contains a Signature HECT Motif, WWE, and a UBA Domain

(A) Schematic representation of the ARF-BP1 polypeptides.
(B) An alignment of the HECT domain of human ARF-BP1 with mouse ARF-BP1 and human E6-AP. Homologous amino acid residues are highlighted in outline and shadow.
(C) ARF-BP1 is widely expressed in different types of human tissue. A multiple tissue Northern filter was hybridized with ARF-BP1 (upper) or actin (lower) cDNA probes.

tated endogenous ARF-BP1 (lane 3, upper panel, Figure 3B); more importantly, ARF was clearly detected in the immunoprecipitations obtained with the α-ARF-BP1 antiserum (lane 3, lower panel, Figure 3B) but not the control IgG (lane 2, lower panel, Figure 3B). Conversely, endogenous ARF-BP1 was readily immuno-precipitated with the ARF-specific antibody (lane 3, Figure 3C), but not with a control antibody (lane 2, Figure 3C). These data indicate that ARF and ARF-BP1 interact both in vitro and in vivo.

The HECT Domain of ARF-BP1 Has a Ubiquitin Ligase Activity that Is Strongly Inhibited by ARF

Although ARF can stabilize p53 by sequestering Mdm2 in the nucleolus (Weber et al., 1999), it is well accepted that ARF can also stabilize p53 by directly inhibiting the enzymatic activity of Mdm2 (Sherr, 2001; Honda and Yasuda, 1999; Midgley et al., 2000). Given that ARF-BP1 contains a HECT motif (Figure 2A), we examined whether ARF can also inhibit the ubiquitin ligase activity of ARF-BP1. To do so, we first tested whether ARF-BP1 displays enzymatic activity in an in vitro assay using purified components. Therefore, the GST-ARF-BP1 (3760–4374) polypeptide, which includes the HECT domain of ARF-BP1, was expressed in bacteria and

purified to near homogeneity. As indicated in Figure 3D, ubiquitin-conjugated forms of ARF-BP1 were readily formed when GST-ARF-BP1 (3760–4374) was incubated in the presence of ubiquitin, E1, and an E2 (UbcH5c) (lane 2). Notably, this activity was strongly repressed by recombinant full-length ARF (lane 3). Moreover, consistent with the binding results (Figure 3A), the evolutionarily conserved N-terminal region of ARF, but not the C-terminal region, inhibited ARF-BP1-mediated auto-ubiquitination (lanes 4 and 5). These data suggest that ARF functions as a potent enzymatic inhibitor of the ARF-BP1 ubiquitin ligase activity.

Inactivation of ARF-BP1 Induces Cell Growth Repression in p53 Null Cells

Although numerous studies have shown that in normal cells ARF stabilizes and activates p53 by inhibiting Mdm2 function, ARF can also inhibit the growth of p53 null cells (Eymin et al., 2003; Yarbrough et al., 2002; Weber et al., 2000). Since we identified ARF-BP1 as the major ARF binding protein from p53 null cells, it is feasible that ARF induces p53-independent growth suppression by inhibiting ARF-BP1 function. To test this hypothesis, we examined whether inactivation of endogenous ARF-BP1 also represses cell growth in p53
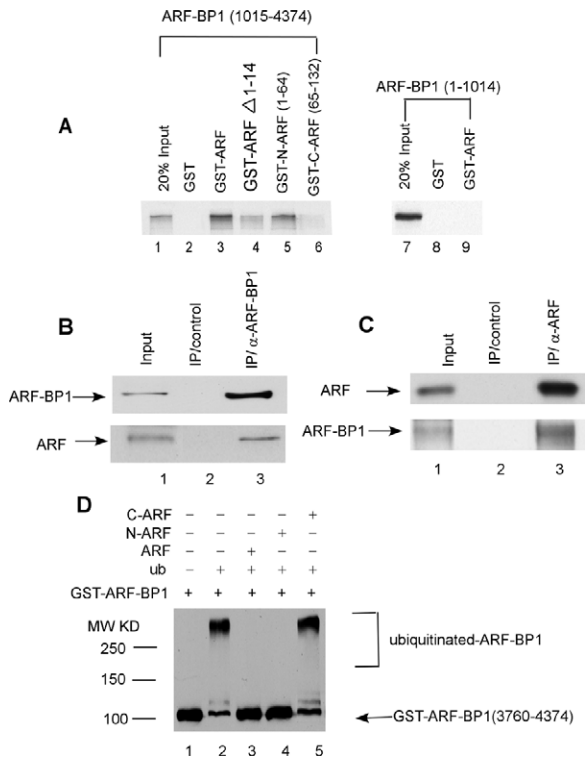
Figure 3. ARF Interacts with ARF-BP1 In Vitro and In Vivo, and ARF-BP1-Mediated Ubiquitin Ligase Activity Is Inhibited by ARF

(A) Direct interactions of ARF-BP1 with GST-ARF. The wild-type GST-ARF full-length protein (GST-ARF) (lanes 3 and 9), the mutant GST-ARF (GST-ARFΔ1–14) (lane 4), the N terminus of ARF protein (1–64) (lane 5), the C terminus of ARF (65–132) (lane 6), or GST alone (lanes 2 and 8) was used in a GST pull-down assay either with in vitro translated ^{35}S-labeled ARF-BP1 (1015–4374) (lanes 1–6), or with in vitro translated ^{35}S-labeled ARF-BP1 (1–1014) (lane 7–9).

(B) Coimmunoprecipitation of ARF with ARF-BP1 from H1299 cells. Western blot analysis of indicated whole-cell extract (WCE) (lane 1) and immunoprecipitates with an ARF-BP1-specific antibody (lane 3) or a control IgG (lane 2) by anti-ARF monoclonal antibody (lower) or anti-ARF-BP1 antibody (top).

(C) Coimmunoprecipitation of ARF-BP1 with ARF from H1299 cells. Western blot analysis of whole-cell extract (WCE) (lane 1) or immunoprecipitates with anti-ARF polyclonal antibody (lane 3) or a control antiserum (lane 2) by a ARF-BP1-specific antibody (lower) or anti-ARF monoclonal antibody (top).

(D)The ubiquitination activity of ARF-BP1 is inhibited by ARF. Western blot analysis of the ubiquitin conjugates by anti-GST antibody. The in vitro ubiquitination assay was set up by incubating GST-ARF-BP1 (3760–4374) with E1, E2 (His-UBCH5a), and ubiquitin (lane 2), or in the presence of GST-ARF (lane 3), GST-NARF (lane 4), or GST-CARF (lane 5), respectively.

null cells in a manner reminiscent of ARF induction. For this purpose, p53 null H1299 cells were transfected with either an ARF-BP1-specific (ARF-BP1-RNAi1) or a control (GFP-RNAi) siRNA. As shown in Figure 4A, the levels of endogenous ARF-BP1 polypeptides were severely reduced after three consecutive transfections (upper panels, lane 3 versus lane 2) with ARF-BP1-RNAi1. As expected, the steady-state levels of p21 and Mdm2, two known transcriptional targets of p53, were unaffected by ARF-BP1 ablation. Strikingly, however,

ARF-BP1-RNAi treatment significantly reduced the growth rate of these cells (Figure 4B), suggesting that ARF-BP1 inactivation induces cell growth repression. Moreover, these cells grew slightly faster when endogenous Mdm2 expression was diminished with RNAi in these cells (Figures 4A and 4B). By monitoring BrdU incorporation (Figure 4C), we again found that ARF-BP1 knockdown inhibits, while Mdm2 knockdown modestly promotes, the growth of p53 null cells. Similar results were also obtained with another p53 null cell line (SaoS-2; Figure S3) and another siRNA (ARF-BP1-RNAi2) that recognizes a different region of the ARF-BP1 mRNA (Figure S4).

Interestingly, ARF-mediated cell growth in p53 null cells is not well characterized. For example, it was initially observed that ARF overexpression induces G1 arrest in p53/Mdm2 double null MEF cells (Weber et al., 2000); however, more recent studies show that ARF expression induces G2/M arrest in a number of p53 null human cell lines (Normand et al., 2005; Eymin et al., 2003). To further analyze the nature of cell growth arrest mediated by ARF-BP1 inactivation in H1299 cells, we first examined the effect of ARF expression in these cells. As shown in Figure 4D, ARF expression induced G2/M accumulation of these cells, but no obvious apoptotic cells (Sub-G1) were observed ("iii" versus "i" in Figure 4D). Strikingly, inactivation of ARF-BP1 by ARF-BP1 RNAi in these cells also led to G2/M arrest at similar levels ("ii" in Figure 4D). Thus, inactivation of ARF-BP1 inhibits the growth of these p53 null cells in a manner reminiscent of ARF induction.

Inactivation of Endogenous ARF-BP1 Stabilizes p53 and Induces p53-Dependent Apoptosis

To investigate the role of the ARF/ARF-BP1 interaction in p53-positive cells, we tested the functional consequences of ARF-BP1 inactivation in cells expressing wild-type p53. Thus, we transfected human osteosarcoma U2OS cells with either an ARF-BP1-specific siRNA (ARF-BP1-RNAi1) or a control siRNA (GFP-RNAi); surprisingly, RNAi-mediated knockdown of ARF-BP1 expression elevated the steady-state levels of endogenous p53 (Figure 5A) and extended the half-life of p53 polypeptides (Figure 5B and Figure S5). Similarly, the expression of p21 and BAX, both known transcriptional targets of p53, was strongly induced by ARF-BP1 inactivation (Figure 5A). Significantly, ARF-BP1 ablation also induced programmed cell death; as shown in Figure 5C, 32.3% of the ARF-BP1-RNAi1-treated U2OS cells underwent apoptosis ("II" in Figure 5C), while no significant apoptosis was observed in the control-transfected cells ("I" in Figure 5C). These data suggest that inactivation of ARF-BP1 stabilizes p53 and activates its mediated functions.

The above results were unexpected given the view that Mdm2 is the primary mediator of ARF-induced p53 activation. To verify the specific effects induced by ARF-BP1 ablation, we performed multiple control experiments. Thus, for example, we were able to knock down endogenous ARF-BP1 expression and again elevate the endogenous levels of p53 by treating cells with a siRNA (ARF-BP1-RNAi 2) that recognizes a different region of the ARF-BP1 mRNA, but not with a point mu-
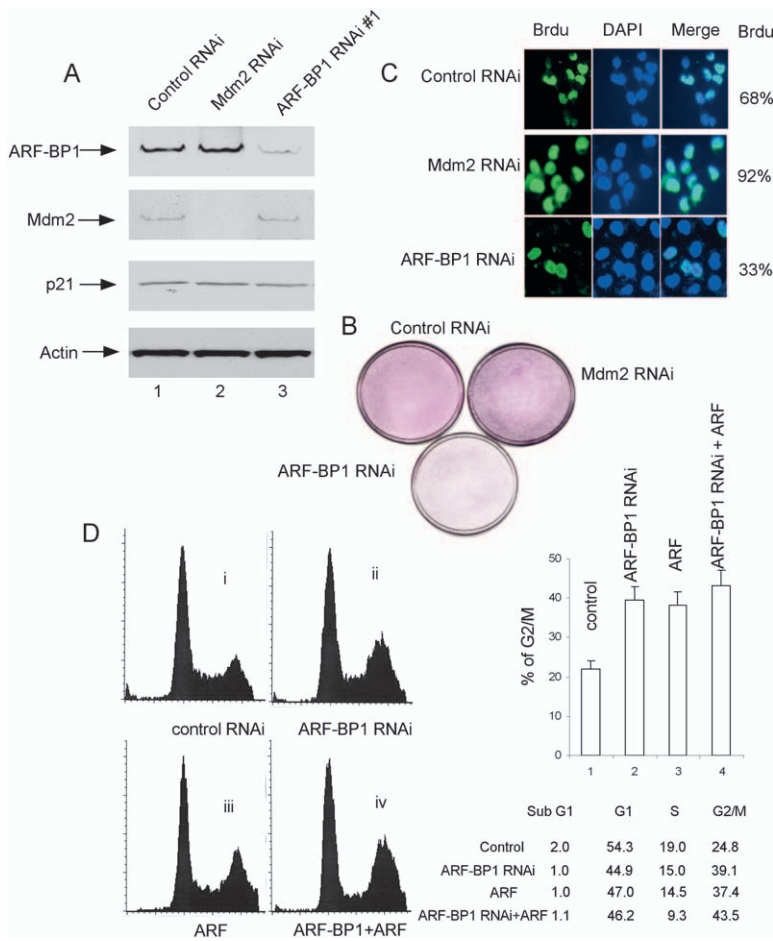
Figure 4. Inactivation of Endogenous ARF-BP1, but Not Mdm2, Induces Cell Growth Arrest in p53 Null H1299 Cells

(A) Endogenous ARF-BP1 and Mdm2 proteins were ablated by RNAi. Western blot analysis of cell extracts of H1299 cells treated with a control RNAi (GFP-RNAi) (lane 1), Mdm2 RNAi (lane 2), or ARF-BP1 RNAi 1 (lane 3) with the antibodies against ARF-BP1, Mdm2, p21, and actin.

(B) Overall cell growth of the H1299 cells treated with a control RNAi (GFP-RNAi), Mdm2 RNAi, or ARF-BP1 RNAi 1. The cells were stained with crystal violet 3 days after siRNA treatment.

(C) The BrdU incorporation of the H1299 cells treated with a control RNAi (GFP-RNAi), Mdm2 RNAi, or ARF-BP1 RNAi 1. The cells were labeled and stained 1 day after RNAi treatment.

(D) Inactivation of ARF-BP1 induces G2M arrest in H1299 cells, similar to overexpression of ARF. Cell cycle profile of control RNAi plus control virus treatment ("i"), ARF-BP1 RNAi ("ii"), adenoviral-ARF treatment ("iii"), and ARF-BP1 RNAi plus adenoviral-ARF treatment.

tant form of the siRNA (ARF-BP1-RNAi 1-mut) (Figure S6; also see Figure S4D). In addition, similar results were obtained using a variety of different cell lines that retain wild-type p53 function, including MCF-7 human breast carcinoma cells (Figure S7), A549 human lung adenocarcinoma cells (Figure S7), and normal human fibroblast cells (NHF-1) (Figure S8).

To further demonstrate the specificity of ARF-BP-RNAi-mediated effects, we performed the rescue experiments. We first made a new expression vector for ARF-BP1, which contains a point mutation at the RNAi1 targeting region [ARF-BP1(R)] (Figure S9A). Thus, this mutant is immune to the effect by the ARF-BP1 RNAi1. To further elucidate the importance of ubiquitin ligase activity of ARF-BP1, we have also made another mutant [ARF-BP1M(R)], in which the conserved cysteine residue at the HECT domain is replaced by alanine (aa 4341 C→A; Figure S9A). By using in vitro ubiquitination assay, we have confirmed that this mutation at the HECT domain abrogates the ubiquitin ligase activity of ARF-BP1 (Figure S9B).

To perform the "rescue experiments," we first conducted the RNAi assay in U2OS cells with ARF-BP1-RNAi1 and then tried to rescue by expressing the ARF-BP1 mutant [ARF-BP1(R)]. As indicated in Figure 5D, after the ARF-BP1 RNAi1 treatment, endogenous p53 was stabilized, and p21 was activated; however, ARF-

BP1(R) expression reversed the effect on the p53 stabilization and p21 induction induced by the ARF-BP1 RNAi1 (lane 3 versus lane 2). Notably, the HECT mutant form [ARF-BP1M(R)], which was expressed at similar levels, failed to rescue the effects (lane 4 versus lane 2) (Figure 5D). We also used this approach for p53-independent function in H1299 cells. Indeed, the cell growth inhibition induced by the ARF-BP1 RNAi1 treatment was rescued by expression of ARF-BP1(R), but not the HECT mutant form [ARF-BP1M(R)] (Figure S10). Thus, these data demonstrate not only the specificity of the ARF-BP1-RNAi-mediated effects but also the importance of the ubiquitin ligase activity in ARF-BP1-mediated functions.

To prove rigorously that these effects of ARF-BP1 are p53 dependent, we performed the siRNA assay in a pair of isogenic human colorectal carcinoma lines that do or do not express wild-type p53 (Bunz et al., 1998). As shown in Figure 5E, when HCT116 parental cells and HCT116 $p53^{-/-}$ cells were subjected to RNAi treatment, ARF-BP1 knockdown led to stabilization of p53 and induction of p21 in the parental cells, but not in the p53 null cells. In contrast, the levels of control proteins such as c-Myc and actin were unaffected by RNAi treatment. Significantly, ARF-BP1 ablation induced apoptosis in parental HCT116 cells, but not in p53 null HCT116 derivatives (Figure S11). As expected, the steady-state
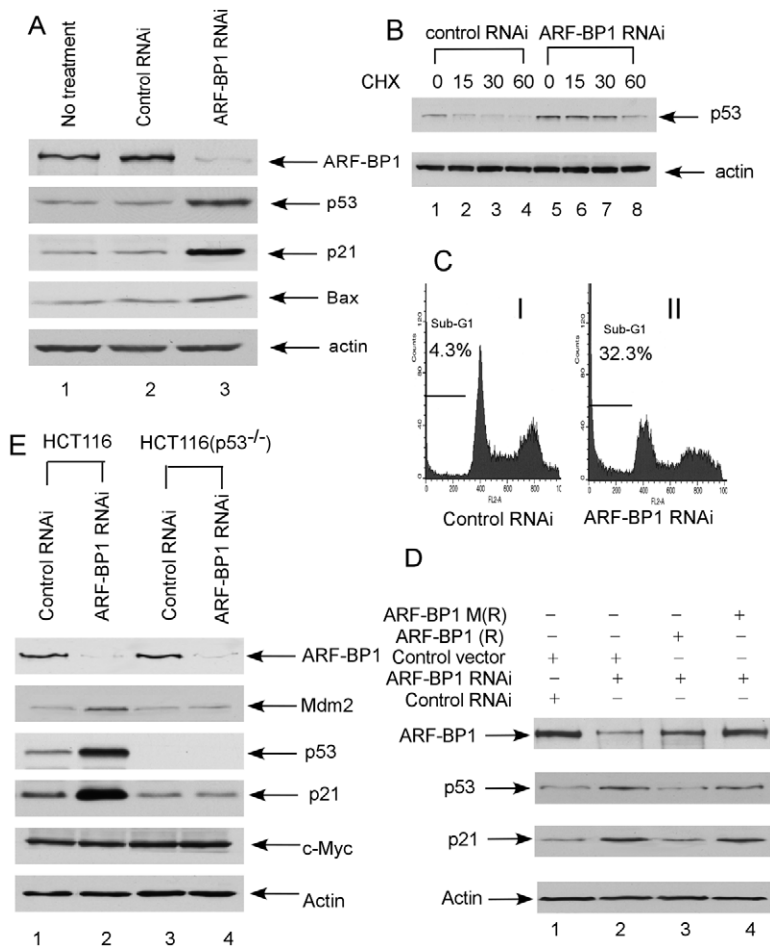
Figure 5. Inactivation of ARF-BP1 Stabilizes p53 and Induces p53-Dependent Apoptosis

(A) Endogenous ARF-BP1 was knocked down by RNAi in human U2OS cells. Western blot analysis of cell extracts of native U2OS cells (lane 1), the U2OS cells treated with a control RNAi (GFP-RNAi) (lane 2), or ARF-BP1 RNAi 1 (lane 3) with the antibodies against ARF-BP1, p53, p21, bax, and actin.

(B) Inactivation of ARF-BP1 extends the half-life of endogenous p53 protein. Western blot analysis of cell extracts with an anti-p53 (DO-1) antibody, from ARF-BP1-RNAi- or control-RNAi-transfected cells, harvested at indicated time points (min) after cyclohexamide (CHX) treatment.

(C) Inactivation of ARF-BP1 induces apoptosis. U2OS cells transfected with either ARF-BP1-RNAi or control-RNAi were analyzed for apoptotic cells (sub-G1) according to DNA content (PI staining).

(D) ARF-BP1-RNAi-mediated effects are reversed by ARF-BP1(R) expression in U2OS cells. Western blot analysis of cell extracts of the U2OS cells treated with a control RNAi (GFP-RNAi) (lane 1), ARF-BP1 RNAi 1 (lane 2), or combination of ARF-BP1 RNAi 1 and ARF-BP1(R) (lane 3), ARF-BP1 RNAi 1, and ARF-BP1M(R) (lane 4) with the antibodies against ARF-BP1, p53, p21, and actin.

(E) Western blot analysis of cell extracts from parental HCT 116 cells (lanes 1 and 2) or *HCT116-p53*^{−/−} cells (lanes 3 and 4) treated with either control RNAi (lanes 1 and 3) or ARF-BP1-RNAi (lanes 2 and 4), with the antibodies against ARF-BP1, Mdm2, p53, p21, Myc, and actin.

levels of Mdm2 were also induced by ARF-BP1 inactivation in the parental cells, consistent with the fact that Mdm2 is a transcription target of p53. Notably, however, Mdm2 levels in the p53 null cells were unaffected by ARF-BP1 ablation (Figure 5E), suggesting that inactivation of ARF-BP1 induces p53 stabilization but has no effect on Mdm2 stabilization. By demonstrating that ARF-BP1 inactivation is sufficient to stabilize and activate p53 in normal cells, these data imply that the ARF/ARF-BP1 interaction may contribute, at least in part, to p53 activation induced by ARF.

ARF-BP1 Directly Binds and Ubiquitinates p53, and ARF-BP1-Mediated Ubiquitination of p53 Is Inhibited by ARF

To elucidate the functional relationship between p53 and ARF-BP1, we examined whether ARF-BP1 can bind p53 in the absence of ARF. As shown in Figure 6A, ^{35}S-labeled ARF-BP1 (1015–4374) strongly bound immobilized GST-p53, but not GST alone (lane 3 versus lane 2). Conversely, no significant binding was detected between ARF-BP1 and GST-Mdm2 (lane 4). We next evaluated the in vivo interaction between endogenous p53 and ARF-BP1 proteins. To this end, cell extracts from U2OS cells were immunoprecipitated with α-ARF-BP1 or with the control IgG. As seen in Figure 6B, p53

was clearly detected in the immunoprecipitates obtained with the α-ARF-BP1 antiserum (lane 3) but not the control IgG (lane 2, lower panels). Conversely, endogenous ARF-BP1 was readily immunoprecipitated with the p53-specific monoclonal antibody DO-1 (lane 3, Figure 7C), but not with a control antibody (lane 2, Figure 6C). These data indicate that p53 can interact directly with the ARF-BP1 protein both in vitro and in vivo.

Since our data show that inactivation of ARF-BP1 induces p53 stabilization, we examined whether ARF-BP1 directly induces p53 ubiquitination in the absence of Mdm2. Thus, Flag-p53 was incubated with GST-ARF-BP1 in the presence of HA-tagged ubiquitin (HA-Ub), E1, and an E2 (UbcH5c). The ubiquitin-conjugated p53 products of the reaction were then immunoprecipitated with Flag/M2 beads and visualized by Western analysis with a p53-specific antibody. As indicated in Figure 6D, high levels of ubiquitinated p53 were generated by ARF-BP1 (lane 2). Significantly, ARF-BP1-mediated p53 ubiquitination was strongly repressed in the presence of ARF (lane 3). Also, consistent with the binding data shown in Figure 3A, the N-terminal region of ARF (N-ARF) retained full inhibition of ARF-BP1-mediated p53 ubiquitination whereas the C-terminal region (C-ARF) showed no effect (lanes 4 and 5). These data suggest
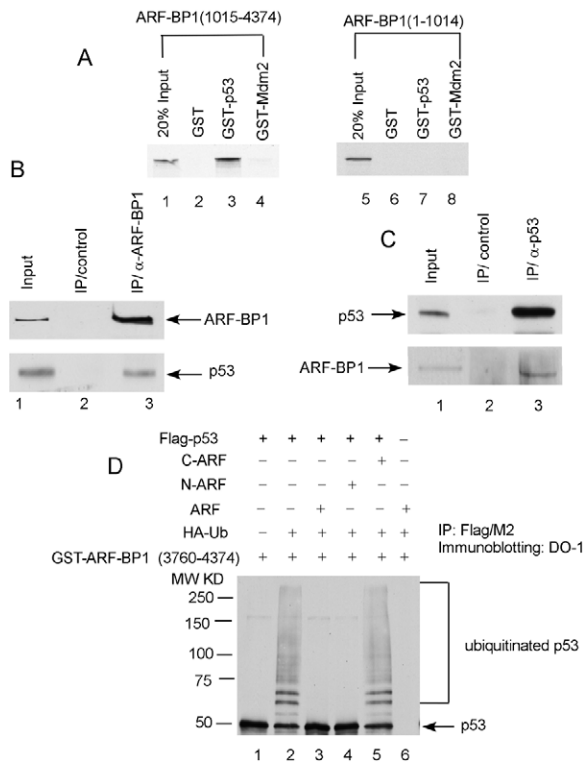
Figure 6. ARF-BP1 Binds and Ubiquitinates p53, and ARF-BP1-Mediated Ubiquitination of p53 Is Inhibited by ARF

(A) Direct interactions of ARF-BP1 with GST-p53. The GST-p53 protein (lanes 3 and 7), GST-Mdm2 (lanes 4 and 8), or GST alone (lanes 2 and 6) was used in a GST pull-down assay with in vitro translated ^{35}S-labeled ARF-BP1 (1015–4374) (lanes 1–4) or ARF-BP1 (1–1014) (lanes 5–8).

(B) Coimmunoprecipitation of p53 with ARF-BP1 from U2OS cells. Western blot analysis of whole-cell extract (WCE) (lane 1) or immunoprecipitates with anti-ARF-BP1-specific antibody (lane 3) or a control IgG (lane 2) by a p53 monoclonal antibody DO-1 (lower) or ARF-BP1-specific antibody (top).

(C) Coimmunoprecipitation of ARF-BP1 with p53 from U2OS cells. Western blot analysis of indicated whole-cell extract (WCE) (lane 1) and immunoprecipitates with a p53 monoclonal antibody DO-1 (lane 3) or control antibody (lane 2) by anti-ARF-BP1-specific antibody (lower) or anti-p53 DO-1 antibody (top).

(D) ARF-BP1-mediated ubiquitination of p53 is inhibited by ARF. After incubation of Flag-p53 with GST-ARF-BP1 (3760–4374) in the presence of E1, E2, and ubiquitin (HA-Ub), the generated p53-ubiquitin conjugates were immunoprecipitated by the Flag/M2 beads and analyzed by Western blot with anti-p53 DO1 antibody. The recombinant bacteria expressed proteins GST-ARF, N-ARF (1–64), or C-ARF (65–132) were added in the reactions shown in lanes 3, 4, or 5, respectively.

that ARF-BP1 is an ubiquitin ligase for p53 and that ARF-BP1-mediated ubiquitination of p53 is repressed by ARF.

ARF-BP1 Is Critical for ARF-Mediated p53 Stabilization in Mdm2 Null Cells

Since ARF-BP1 binds and ubiquitinates p53 in the absence of Mdm2, it is conceivable that the ARF/ARF-BP1 interaction stabilizes p53 in an Mdm2-independent manner. To test this hypothesis, we first examined
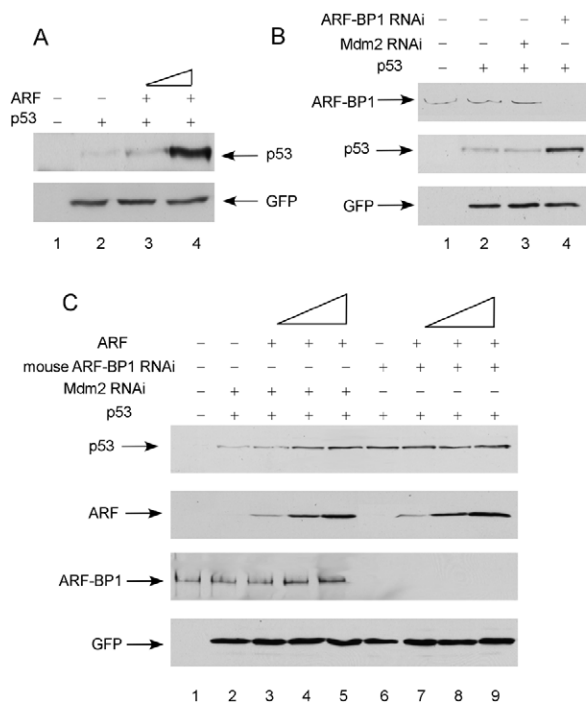
Figure 7. ARF Induces p53 Stabilization in an Mdm2-Independent Manner, and ARF-BP1 Is Critical for ARF-Mediated p53 Stabilization in Mdm2 Null Cells

(A) ARF stabilizes p53 in Mdm2 null cells. Western blot analysis of cell extracts from MEF p53/Mdm2-double null cells transfected with expression vectors of p53 and ARF with a p53 antibody (DO-1).

(B) Inactivation of ARF-BP1 stabilizes p53 in Mdm2 null cells. Western blot analysis of cell extracts from MEF p53/Mdm2 double null cells transfected with the p53 expression vector together with either ARF-BP1 RNAi or Mdm2 RNAi, by a p53 antibody (DO-1).

(C) ARF-BP1 is required for ARF-mediated p53 stabilization in Mdm2 null cells. Western blot analysis of cell extracts from MEF p53/Mdm2 double null cells transfected with expression vectors of p53 and ARF, together with either ARF-BP1 RNAi or Mdm2 RNAi, by anti-p53 (DO-1), anti-ARF, anti-ARF-BP1, and anti-GFP antibodies.

whether ARF expression induces p53 stabilization in Mdm2 null cells. To this end, we transfected p53/Mdm2 double null MEF cells with expression vectors encoding p53 alone, or both p53 and ARF; indeed, p53 protein levels were significantly elevated in these cells by ARF overexpression (Figure 7A), indicating that ARF can stabilize p53 in an Mdm2-independent manner.

To verify the role of endogenous ARF-BP1 in p53 degradation in the absence of Mdm2, we examined whether inactivation of ARF-BP1 is sufficient to stabilize p53 in Mdm2 null cells. For this purpose, Mdm2/p53 double null cells were cotransfected with a p53 expression vector and siRNAs specific for either ARF-BP1 or Mdm2. Significantly, ablation of endogenous ARF-BP1 expression in the cells caused a marked stabilization of p53 (Figure 7B and Figure S12). As expected, treatment with Mdm2-specific siRNAs had no effect on p53 levels in Mdm2/p53 null cells (lane 3, Figure 7B), confirming the specificity of p53 stabilization by ARF-BP1 inactivation.

Finally, to provide direct evidence that ARF-BP1 is involved in the Mdm2-independent p53 stabilization induced by ARF, we examine whether ARF-BP1 is required for ARF-mediated p53 stabilization in Mdm2 null cells. Thus, p53/Mdm2 double null cells were cotransfected with ARF-BP1-specific siRNAs and expression vectors encoding p53 and ARF. As indicated in Figure 7C, the p53 stabilization induced by ARF was clearly attenuated in ARF-BP1 knockdown cells (lanes 6–9), suggesting that ARF-BP1 is critical for ARF-mediated p53 stabilization in these cells. In contrast, ARF-mediated p53 stabilization was intact in cells treated with Mdm2-specific siRNAs (lanes 2–5, Figure 7C). Taken together, these results indicate that ARF-BP1 is critical for the ARF-mediated, Mdm2-independent stabilization of p53.

Discussion

The present data demonstrate a critical role of ARF-BP1 in mediating both the p53-dependent and p53-independent functions of ARF. Here, we show that (1) ARF-BP1 is the major component of ARF-containing nuclear complexes in human cells; (2) ARF-BP1 interacts with both ARF and p53, but not with Mdm2; (3) ARF-BP1 is widely expressed and contains signature motifs (HECT, WWE, and UBA) commonly associated with protein ubiquitination; (4) ARF-BP1 has an intrinsic ubiquitin ligase activity, and this activity is strongly repressed by ARF; (5) inactivation of endogenous ARF-BP1, but not Mdm2, induces cell growth repression in p53 null cells in a manner reminiscent of ARF induction; (6) ARF-BP1 catalyzes ubiquitination of p53 directly, and inactivation of endogenous ARF-BP1 in p53 wild-type cells stabilizes p53 and activates p53 function; and (7) ARF-BP1 is required for ARF-mediated p53 stabilization in Mdm2 nulls. Since ARF can stabilize p53 in an Mdm2-independent manner, and inactivation of ARF-BP1 by ARF also induces growth arrest in p53 null cells, our study significantly modifies the current view of how ARF mediates its tumor suppressor function in vivo.

The Role of ARF-BP1 in ARF-Mediated Activation of p53

While it is widely accepted that ARF is a bona fide tumor suppressor (Sherr, 2001; Sharpless and DePinho, 2004), the first clue that it can activate the p53 pathway emerged from tissue culture experiments showing that p53 stabilization is crucial for ARF-mediated function (Kamijo et al., 1997). At the time, the role of Mdm2 in ubiquitination and degradation of p53 had recently been discovered (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). Based primarily on results derived from experiments involving protein overexpression, this seemingly obvious connection between ARF and Mdm2 was soon regarded as the primary pathway for ARF-mediated p53 activation (Sherr, 2001).

However, several lines of evidence indicate that ARF-mediated activation of p53 is more complicated than the simple ARF-Mdm2-p53 model. First, the ARF/ Mdm2 interaction was originally defined in experiments based on Mdm2 overexpression; nevertheless, the steady-state levels of Mdm2 in normal cells are low, and an interaction between the endogenous ARF and Mdm2 polypeptides in normal cells has not yet been well characterized. Second, while the low levels of Mdm2 that are commonly observed in normal cells preferentially catalyze monoubiquitination of p53 (Li et al., 2003), recent studies from Lane's group indicate that ARF can only block p53 polyubiquitination but is incapable of inhibiting Mdm2-mediated monoubiquitination of p53 in vivo (Xirodimas et al., 2001b). These studies raise a critical question: how does ARF efficiently stabilize p53 in the cells where the levels of Mdm2 are low and Mdm2 is not solely responsible for p53 degradation? Our results on ARF-BP1 implicate a novel Mdm2-independent pathway for ARF-mediated p53 activation. ARF-BP1 was identified as a major component of ARF-containing protein complexes, and the interaction between endogenous ARF-BP1 and ARF proteins is easily detected in unstressed cells.

Several recent studies support the notion that p53 degradation is mediated by both Mdm2-dependent and Mdm2-independent pathways in vivo (Leng et al., 2003; Dornan et al., 2004). Interestingly, by using RNAi knocking-down approaches, we tended to examine the differential effects on p53 stabilization by these known E3 ligases of p53. As expected, inactivation of Mdm2 promoted p53 stabilization, while inactivation of either COP1 or Pirh2 also modestly stabilized p53. Notably, inactivation of ARF-BP1 strongly induced p53 stabilization and activated p53-mediated transcription (Figure 8A); the levels of p21 and Bax induction induced by ARF-BP1 RNAi were higher than the levels induced by other types of E3 siRNAs for p53 (Figure 8A) and very close to the effects by ARF overexpression (Figure S13). These data indicate that ARF-BP1 is a major ubiquitin ligase of p53 in human cells and, more importantly, is also a key target for ARF-mediated tumor suppressor function.

The existence of two distinct pathways for ARF-mediated p53 activation, one based on the ARF-BP1 ubiquitin ligase and another based on the Mdm2 ubiquitin ligase, allows for more versatile control of p53 functions (Figure 8B) but also raises the question regarding their biological significances. For example, the critical role of Mdm2 in tumorigenesis is well established. Gene amplification and protein overexpression of Mdm2 are found in various types of tumors (Michael and Oren, 2003). Thus, the ARF-Mdm2 interaction might be particularly important in the cells expressing high levels of Mdm2. Through a database search, we found that the gene encoding ARF-BP1 is localized on chromosome Xp11.22. Interestingly, we also found that ARF-BP1 is highly expressed in 80% (16/20) of breast cancer cell lines, while the expression level of ARF-BP1 in normal breast cells (MCF-10A) is low (Figure 8C), suggesting a potential role of ARF-BP1 in breast cancer tumorigenesis.

The Role of ARF-BP1 in p53-Independent ARF Tumor Suppression

Although its role in activating p53 is well accepted, ARF is also found to be mutated or downregulated in tumors that lack functional p53 (Sherr, 2001). This suggests
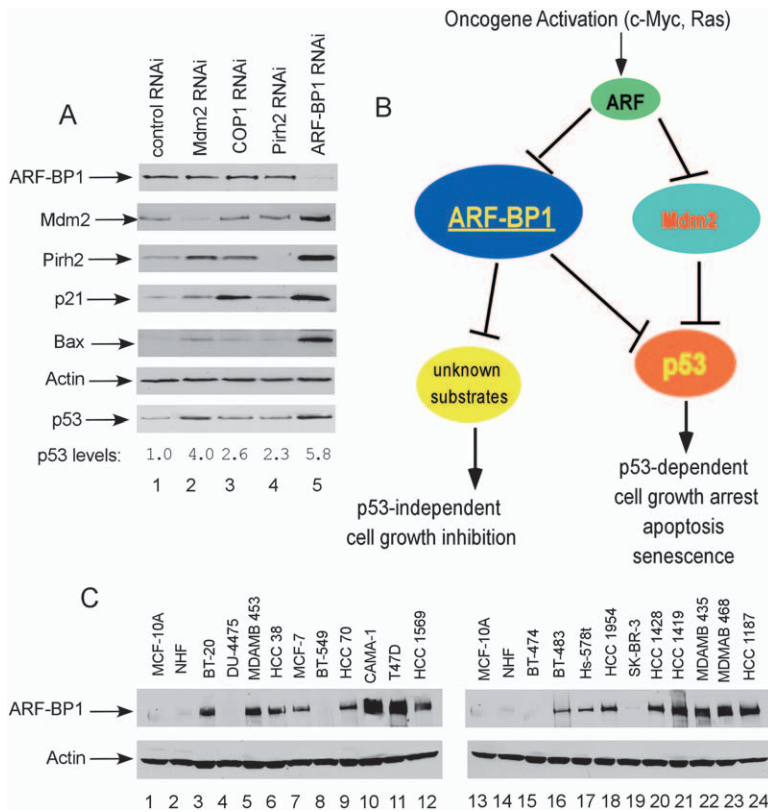
Figure 8. ARF-BP1 Is a Critical Mediator of ARF Tumor Suppressor Function

(A) Reduction of ARF-BP1 has the most significant effect on p53 levels when compared to the known E3 ligases for p53, including Mdm2, COP1, and Pirh2. Western blot analysis of cell extracts from U2OS treated with control RNAi (lanes 1), Mdm2 RNAi (lane 2), COP1 RNAi (lane 3), Pirh2 RNAi (lane 4), and ARF-BP1 RNAi 1 (lane 5), with the antibodies against ARF-BP1, Mdm2, Pirh2, p53, p21, Bax, and actin. The COP1 antibody was not available.

(B) A model for cooperative controls of the p53-dependent and p53-independent functions of ARF by ARF-BP1 and Mdm2. See text for details.

(C) ARF-BP1 expressions in breast cancer cell lines. Western blot analysis of cell extracts from a number of breast cancer cell lines compared with the normal breast cell line MCF-10A as well as the normal human fibroblast NHF cell line by anti-ARF-BP1-specific antibody and anti-actin antibody.

that the p53-independent functions of ARF are also critical for its tumor suppression activity. Consistent with this notion, a number of studies indicate that ARF can repress cell growth in a p53-independent manner (Weber et al., 2000; Normand et al., 2005; Rocha et al., 2003; Itahana et al., 2003; Eymin et al., 2003; Yarbrough et al., 2002; Korgaonkar et al., 2002). Based on our observation that ARF-BP1 is the major binding partner of ARF in p53 null cells, we propose that ARF-BP1 is a critical mediator of p53-independent ARF functions. This hypothesis is further supported by the fact that inactivation of ARF-BP1, but not Mdm2, induces cell growth repression in p53 null cells in a manner reminiscent of ARF induction.

The precise mechanism by which ARF-mediated regulation of ARF-BP1 leads to p53-independent cell growth arrest needs future investigation. Since ARF-BP1 is a bona fide ubiquitin ligase, it is likely that the p53-independent functions of ARF involve unidentified enzymatic substrates of ARF-BP1 (Figure 8B). In the light of recent studies showing that nucleophosmin/B23 and certain ribosomal subunits are involved in the regulation of ARF-mediated ribosomal RNA processing (Itahana et al., 2003; Bertwistle et al., 2004), it will be interesting to examine whether ARF-BP1 is directly involved in regulating B23 function or ribosomal RNA processing. Moreover, since the ARF pathway is intimately linked with oncogene activation in vivo (Sherr, 2001; Sharpless and DePinho, 2004), it will be intriguing to know whether the ARF/ARF-BP1 interaction, as well as the ubiquitin ligase activity of ARF-BP1, is regulated by oncogene activation or other forms of stress.

Moreover, ARF-BP1 may prove to be an especially valuable target for therapeutic intervention. Agents that activate the p53 tumor suppression pathway, including Nutlin, a small molecule antagonist of Mdm2 (Vassilev et al., 2004), are currently being tested for their potential in cancer therapy. However, the utility of these agents is limited to tumors that maintain a functional p53 pathway, a significant restriction given that p53 mutations are found in more than 50% of human cancers. In contrast, since ARF-BP1 inactivation induces growth arrest in p53 null cells and also activates p53-dependent apoptosis in p53 wild-type cells, inhibitors of ARF-BP1 should suppress tumor cell growth regardless of p53 status.

Experimental Procedures

Plasmids

To clone the cDNA of ARF-BP1, five overlapping cDNA sequences that cover the full-length ARF-BP1 were amplified by PCR from Marathon-Ready HeLa cDNA (Clontech, BD Biosciences) and subcloned into pcDNA3.1/V5-His-Topo vector (Invitrogen). After sequence verification, the cDNA sequences were assembled and further cloned into expression vectors. To prepare mutant constructs [ARF-BP1(M), ARF-BP1(R), ARF-BP1M(R)], cDNA sequences corresponding to different regions were amplified by PCR from above constructs using QuikChange Site-Directed Mutagenesis Kit (Stratagene) and subcloned into full-length ARF-BP1 using specific restriction enzymes. For the HA-ARF-Flag construct, the HA and Flag sequence were introduced to the N terminus and C terminus ARF, respectively, by PCR and subcloned into the pCIN4 vector.

Purification of ARF Complexes from Human Cells

The epitope-tagging strategy to isolate ARF-containing protein complexes from human cells was performed essentially as pre-

viously described with some modifications (Gu et al., 1999; Luo et al., 2000; Nikolaev et al., 2003). In brief, to obtain an HA-ARF-Flag-expressing cell line, p53 null H1299 cells were transfected with pCIN4-HA-ARF-Flag and selected for 2 weeks in 1 mg/ml G418. The stable cell lines were chosen to expand for complex purification if the expression levels of the ectopic ARF protein were close to the levels of endogenous protein. Thus, the cells were grown in DMEM with 10% fetal bovine serum and harvested near confluence. The cell pellet was then suspended in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and protein inhibitor mixture). The cells were allowed to swell on ice for 15 min, after which 10% NP40 was added to a final concentration of 0.5%. The tube was vigorously vortexed for 1 min. The homogenate was centrifuged for 10 min at 4000 rpm. The nuclear pellet was resuspended in ice-cold buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and protein inhibitor mixture), and the tube was vigorously rocked at 4°C for 45 min. The nuclear extract was diluted with buffer D (20 mM HEPES [pH 7.9], 1 mM EDTA) to the 100 mM final NaCl concentration and ultracentrifuged at 25,000 rpm for 2 hr at 4°C. After being filtered with 0.45 μm syringe filters, the supernatants were used as nuclear extracts for immunoprecipitations by anti-Flag antibody-conjugated M2 agarose. The bound polypeptides eluted with the Flag peptide were further affinity purified by anti-HA antibody-conjugated agarose. The final elutes from the HA-beads with HA peptides were resolved by SDS-PAGE on a 4%–20% gradient gel for silver staining or colloidal blue staining analysis. Specific bands were cut out from the gel and subjected to mass spectrometry peptide sequencing.

Ablation of Endogenous ARF-BP1 by RNAi in Human Cells
p53 null cell lines (H1299 and Saos-2) and p53-expressing cells (U2OS, MCF-7, NHF-1, and A549) were maintained in DMEM medium supplemented with 10% fetal bovine serum. The HCT116 and *HCT116-p53$^{-/-}$* cell lines were kindly provided by B. Vogelstein's lab. The RNAi-mediated ablation of endogenous ARF-BP1 was performed essentially as previously described (Elbashir et al., 2001; Nikolaev et al., 2003). A 21 nucleotide siRNA duplex with 3′dTdT overhangs corresponding to ARF-BP1 mRNA (ARF-BP1 1) (AAU UGCUAUGUCU CUGGGACA) was synthesized (Dharmacon). The same sequence (ARF-BP1 1 mutant) with two nucleotides changed (AAUUGCCAUGUAUCUGGGACA) was used as a specific RNAi control. The FACS analysis data represent the mean of three experiments with standard deviations indicated. RNAi transfections were performed using Oligofectamine reagent. The siRNAs of COP1 and Pirh2 were used as previously reported (Dornan et al., 2004).

Supplemental Data
The Supplemental Data include Supplemental Experimental Procedures and thirteen figures and can be found with this article online at http://www.cell.com/cgi/content/full/121/7/1071/DC1/.

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Accession Numbers

The GenBank accession number for the human ARF-BP1 sequence is AY772009, and the number for the mouse ARF-BP1 sequence is AY772010.