Inactivation of arf-bp1 Induces p53 Activation and Diabetic Phenotypes in Mice

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Background: ARF-BP1 is involved in Mdm2-independent p53 degradation.

Results: Inactivation of arf-bp1 in mice resulted in p53 activation and embryonic lethality. Inactivation of arf-bp1 in pancreatic β-cells resulted in diabetes, which was partially rescued by loss of p53.

Conclusion: p53 is critically regulated by ARF-BP1 in vivo and in β-cells.

Significance: ARF-BP1 is important for maintaining pancreatic β-cell homeostasis in aging mice.

It is well accepted that the Mdm2 ubiquitin ligase acts as a major factor in controlling p53 stability and activity in vivo. Although several E3 ligases have been reported to be involved in Mdm2-independent p53 degradation, the roles of these ligases in p53 regulation in vivo remain largely unknown. To elucidate the physiological role of the ubiquitin ligase ARF-BP1, we generated arf-bp1 mutant mice. We found that inactivation of arf-bp1 during embryonic development in mice resulted in p53 activation and embryonic lethality, but the mice with arf-bp1 deletion specifically in the pancreatic β-cells (arf-bp1FL/FL/RIP-cre) were viable and displayed no obvious abnormality after birth. Interestingly, these mice showed dramatic loss of β-cells as mice aged, and >50% of these mice died of severe diabetic symptoms before reaching 1 year of age. Notably, the diabetic phenotype of these mice was largely reversed by concomitant deletion of p53, and the life span of the mice was significantly extended (p53FL/FL/arf-bp1FL/FL/RIP-cre). These findings underscore an important role of ARF-BP1 in maintaining β-cell homeostasis in aging mice and reveal that the stability of p53 is critically regulated by ARF-BP1 in vivo.

The p53 tumor suppressor acts as the major sensor for a regulatory circuit that monitors signaling pathways from diverse sources, including DNA damage, oncogenic events, ribosomal stress, and other abnormal cellular processes (1–4). Although p53 mutations have been documented in more than half of human tumors, defects in other key components of the p53 pathway are frequently observed in tumor cells that retain wild type p53. Thus, inactivation of p53 appears to be a common, if not universal, feature of human cancer. It is well accepted that Mdm2 plays a major part in the scope of inhibiting p53 activities in cancer cells. Mdm2, a RING finger oncoprotein, acts as a specific E3 ubiquitin ligase in p53 degradation. The critical role of Mdm2 in regulating p53 is best illustrated by studies carried out in mice where deletion of p53 was shown to rescue completely the embryonic lethality caused by the loss of Mdm2 function (5–7). Although numerous studies validate the crucial role of Mdm2 in regulating p53 stability, p53 still undergoes proteasome-mediated degradation in Mdm2-null cells (8). Accumulating evidence indicates that Mdm2-independent mechanisms are also involved in tissue-specific and temporal control of the stability and function of p53 in vivo. Indeed, the recently discovered E3 ligases COP1 (9), Pth2 (10), Arf-BP1 (11), and others have clearly been shown to contribute to the efficient control of p53 levels in tissue culture and in vitro biochemical experiments. Additionally, USP4 inhibits p53 activity indirectly through regulation of ARF-BP1 (12). Thus, both Mdm2-dependent and Mdm2-independent mechanisms are required to tightly regulate p53 function in vivo.

ARF-BP1, also known as MULE/HectH9/HUWE1, was originally identified as a major binding protein associated with ARF tumor suppressor. The ARF tumor suppressor acts as a key sensor of hyperproliferative signals such as those emanating from the Ras and Myc oncoproteins (13). Numerous studies indicate that ARF suppresses aberrant cell growth in response to oncogene activation by activating the p53 pathway (14). The ARF induction of p53 appears to be mediated through Mdm2 because overexpressed ARF interacts directly with Mdm2 and inhibits its ability to promote p53 degradation (15). Interestingly, ARF also has tumor suppressor functions that do not depend on p53 or Mdm2. ARF-BP1 harbors a signature HECT (homolog to E6-AP C terminus) motif, and its ubiquitin ligase activity is inhibited in the presence of ARF. Notably, inactivation of ARF-BP1, but not Mdm2, suppresses the growth of p53-null cells in a manner reminiscent of ARF induction. Indeed, several recent studies showed that ARF-BP1 is capable of ubiquitininating several p53-independent targets, such as Mcl-1, N-Myc, and Cdc6, leading to diverse functions (16–21). Surprisingly, in p53 wild-type cells, ARF-BP1 inactivation serves to stabilize the p53 polypeptide and activates p53 function. We further show that ARF-BP1 directly binds and ubiquitinates p53 and that inactivation of endogenous ARF-BP1 is crucial for...
ARF-mediated p53 stabilization in Mdm2-null cells (11).
Together, these studies indicate that ARF-BP1 is a critical mediator of both the p53-independent and p53-dependent tumor suppressor functions of ARF. Nevertheless, it remains unclear whether ARF-BP1 is indeed required in regulating p53 in vivo, and the physiological role of ARF-BP1 also needs to be further elucidated.

To elucidate the physiological roles of ARF-BP1, we have generated arf-bp1 conditional knock-out mice to study the effects of ARF-BP1 ablation on mouse development and on mouse pancreatic β-cell functions. Because p53 activation is difficult to detect in pancreatic β-cells, we speculate that p53 is potentially tightly controlled by multiple E3 ubiquitin ligases, including ARF-BP1. Moreover, studying ARF-BP1 in β-cells could potentially uncover p53-independent functions of ARF-BP1, if p53 were indeed greatly suppressed in β-cells. Deletion of arf-bp1 during early embryonic development in mice resulted in embryonic lethality around embryonic day 14.5 (E14.5).2 Furthermore, deletion of arf-bp1 specifically in pancreatic β-cells caused dramatic loss of β-cells and development of age-dependent diabetes in mice. Notably, concomitant deletion of p53 effectively diminished the diabetic phenotype and also significantly elongated the life span of these arf-bp1 mutant mice, suggesting an important role of ARF-BP1 in maintaining β-cell homeostasis through suppressing p53 functions. These results indicate ARF-BP1 as a critical regulator of β-cell homeostasis in aging mice and also suggest that p53 is a potential target in therapeutic treatment of diabetes.

**EXPERIMENTAL PROCEDURES**

Generation of arf-bp1 Conditional Knock-out Mice—To construct the arf-bp1 gene targeting vector, a BAC clone (RP22-407A2) containing the 5’ portion of arf-bp1 was screened from mouse the RP22 BAC genomic library (CHORI, Oakland, CA). We decided to delete exon 11 of arf-bp1 (Ensembl), leading to a translational reading frameshift and loss of >90% of ARF-BP1 protein, including the HECT E3 ubiquitin ligase domain at the C terminus. The targeting vector was constructed by recombineering as described previously (22), which contained a 7-kb 5’ region of exon 11 and 4-kb 3’ region of exon 11 of arf-bp1. As indicated in the diagram (Fig. 1A), loxP sites were inserted in the introns flanking exon 11, to permit deletion of exon 11 upon expression of Cre recombinase. The targeting vector was linearized and electroporated into ES cells. After selection in the

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Mouse Conditional Knock-out of arf-bp1

In this study, we aimed to generate conditional knock-out mice for the arf-bp1 gene, which is located on the X chromosome. The gene encodes ARF-BP1, a protein involved in the control of cell growth and differentiation. To achieve this, we used a conditional knock-out approach involving homologous recombination in mouse embryonic stem (ES) cells. The ES cells were targeted to delete exon 11 of the arf-bp1 gene, which is flanked by loxP sites (floxed) to allow for Cre-mediated excision in vivo.

**Generation of arf-bp1 Conditional Knock-out Mice**

The arf-bp1 gene was targeted in ES cells by homologous recombination. The targeted ES cell clones were identified by Southern blotting using PstI-digested ES cell genomic DNA and a 3′ probe, amplified with a 5′ primer of cctgataaattcctgag and 3′ primer of cctcataacgcaagcggattaa. The targeted ES cell clones were subsequently injected into blastocysts to generate chimeras, from which the exon 11-floxed allele was transmitted through the germ line. Initially, genotyping was determined by Southern blotting (Fig. 1B). Subsequently, it was determined by PCR using primers of WT 5′ primer cctcataacgcaagcggattaa and Neo primer gttattaattcctgagcat to detect the wild-type allele and mutant allele simultaneously (Fig. 1C). The arf-bp1 conditional knock-out mice were kindly provided by Dr. Thomas Ludwig, Columbia University (23). Maintenance and experimental protocols using mice were approved by Institutional Animal Care and Use Committee (IACUC) of Columbia University.

**Analysis of arf-bp1 Conventional Knock-out Mice**

Breeding between arf-bp1FL/YFL/Y female mice and Rosa26-cre homozygote male mice were set up to generate arf-bp1 knock-out mice. The resulting offspring should have equal numbers of arf-bp1+/+ and arf-bp1−/−, among which arf-bp1+/− represents heterozygote embryos, and arf-bp1−/− represents knock-out embryos. No viable arf-bp1 knock-out mice were obtained postnatally, indicating that arf-bp1 knock-out mice were embryonic lethal. Subsequently, phenotypic analysis was done using embryos collected from timed pregnancy at different stages of gestation. Embryos were fixed in PBS-buffered 4% paraformaldehyde overnight and embedded in paraffin. Serial 5-μm sagittal sections were collected and stained by hematoxylin and eosin according to standard procedures. The sections were also immunostained using antibodies against ARF-BP1 (ureb1; Bethyl), p53 (CM5; Novoceastra), and Cleaved Caspase3 (Cell Signaling), followed by counterstaining using hematoxylin.

**Histology and Immunohistochemical Analysis of Islets**

Mouse pancreases were collected, fixed in 4% paraformaldehyde overnight, and embedded in paraffin. Sections of 5 μm were prepared and immunostained according to standard procedures, using antibodies against ARF-BP1 (Bethyl), insulin (Dako), and glucagon (Dako).

**Determination of Blood Glucose and Insulin and Glucose Tolerance Test (GTT)**

The arf-bp1 conditional knock-out mice were back-crossed to C57BL/6j to produce a congenic strain for the study of ARF-BP1 functions in β-cells. The p53 conditional knock-out mice were kindly provided by Dr. Thomas Ludwig, Columbia University (23). Maintenance and experimental protocols using mice were approved by Institutional Animal Care and Use Committee (IACUC) of Columbia University.

**RESULTS**

**Generation of arf-bp1 Conditional Knock-out Mice**

The mouse arf-bp1 gene is located on the X chromosome and contains 83 exons in a span of more than 100 kb of genomic DNA (Ensembl). Because of the location and the size of arf-bp1, we decided to create a conditional knock-out allele of arf-bp1, in which exon 11 is flanked by loxP sites (floxed) to permit deletion of exon 11 upon Cre expression (Fig. 1A).

**Histology and Immunohistochemical Analysis of Islets**

Mouse pancreases were collected, fixed in 4% paraformaldehyde overnight, and embedded in paraffin. Serial 5-μm sagittal sections were collected and stained by hematoxylin and eosin according to standard procedures. The sections were also immunostained using antibodies against ARF-BP1 (ureb1; Bethyl), p53 (CM5; Novoceastra), and Cleaved Caspase3 (Cell Signaling), followed by counterstaining using hematoxylin.

**Determination of Blood Glucose and Insulin and Glucose Tolerance Test (GTT)**

The arf-bp1 conditional knock-out mice were back-crossed to C57BL/6j at least 6 times before crossing with transgenic RIP-Cre (rat insulin promoter controlled cre) mice to generate arf-bp1FL/Y/RIP-cre mice (24, 25). Because arf-bp1 is located on the X chromosome, only the male mice (arf-bp1FL/Y and arf-bp1FL/Y/RIP-cre) were used in the subsequent study. The mice were maintained at a 12-h light/dark cycle. The fasting glucose levels and the fasting insulin levels were determined after the mice were fasted overnight (16 h). The fed glucose levels were measured using mice fed at libitum. The fed insulin levels were determined after mice were first fasted overnight and then fed at libitum for 4 h to maximize insulin secretion. Blood glucose was measured using blood from tail vein with OneTouch UltraMini glucometer (LifeScan; Johnson & Johnson). The blood insulin levels were determined using an Insulin ELISA kit (Millipore) according to the manual provided by the manufacturer. The GTT was done using mice fasted overnight before injecting 2 g of glucose/kg of body weight into the peritoneal cavity. Blood glucose levels were then determined at 15-, 30-, 60-, and 120-min time points after the glucose injection.

**Deletion of arf-bp1 in Mouse Embryonic Fibroblasts (MEFs)**

Using Adenovirus Expressing Cre Recombinase-arf-bp1FL/Y—MEFs were prepared from E13.5 embryos and cultured in DMEM containing 10% FBS. To delete arf-bp1, 2.5 × 10⁵ of cells were mixed with either control adenovirus expressing GFP (Ad-CMV-GFP; Vector Biolaboratories) or adenovirus expressing Cre (Ad-CMV-Cre; Vector Biolaboratories) at a multiplicity of infection of 200 before being plated onto a 6-well plate. Cells were harvested after 3 days, and protein extracts were prepared in radioimmunoprecipitation assay buffer and analyzed by Western blotting. The Western blot was probed with antibodies against ARF-BP1 (Bethyl), p53 (CM5; Novoceastra), p21 (Santa Cruz Biotechnology), BAX (Santa Cruz Biotechnology), and β-actin (Sigma).

**RESULTS**

**Generation of arf-bp1 Conditional Knock-out Mice**

The mouse arf-bp1 gene is located on the X chromosome and contains 83 exons in a span of more than 100 kb of genomic DNA (Ensembl). Because of the location and the size of arf-bp1, we decided to create a conditional knock-out allele of arf-bp1, in which exon 11 is flanked by loxP sites (floxed) to permit deletion of exon 11 upon Cre expression (Fig. 1A). After gene targeting in mouse embryonic stem cells, 2 of 200 clones screened were identified to have correct homologous recombination events by Southern blotting. Subsequently, these clones were injected into mouse blastocysts to derive chimeras, from which germ line transmission of the targeted allele was accomplished. Subsequently, homozygote conditional knock-out males (arf-bp1FL/Y) and females (arf-bp1FL/FL) were obtained through breeding, which were grossly normal and fertile, suggesting that floxing exon 11 has no discernible effects on arf-bp1 transcription.

**arf-bp1 Knock-out Mice Were Embryonic Lethal**

To understand the impacts of knock-out of arf-bp1 in vivo, we generated arf-bp1 knock-out mice (arf-bp1−/−) through breeding between arf-bp1FL/Y heterozygote conditional knock-out female mice and Rosa26-cre homozygote male mice. Because of constitutive Cre expression from the Rosa26-cre allele, mice with the genotype of arf-bp1FL/Y/Rosa26-cre were considered as arf-bp1 knock-out mice (arf-bp1−/−). There were 47 wild-type offspring at weaning age (Table 1), and no arf-bp1 homozygote knock-out mice were obtained postnatally (23 were expected), suggesting that arf-bp1 knock-out mice were embryonic lethal. Furthermore, the number of arf-bp1 heterozygote knock-out (arf-bp1+/−) mice (8 were observed) was underrepresented (23 were expected), presumably due to random X chromosome inactivation of the remaining wild-type allele. To analyze the phenotypes of arf-bp1 knock-out mice, embryos from E11.5 to E17.5 were collected, from which arf-
bp1 knock-out embryos were identified at all stages during development (Table 1). Although arf-bp1 knock-out embryos at E12.5 (Fig. 2D) appeared indistinguishable from wild-type embryos (Fig. 2A), all of the arf-bp1 knock-out embryos displayed hemorrhage in the abdominal region by E14.5 (Fig. 2E), followed by growth impairment, necrosis, and eventual death of the embryos (Fig. 2F). Age-matched control embryos are shown (Fig. 2, B and C). These results confirmed that arf-bp1 knock-out mice were embryonic lethal during late stage of embryonic development.

Ablation of arf-bp1 in Mouse Resulted in p53 Activation—To explore the molecular mechanism for the embryonic lethality in arf-bp1 knock-out mice, sagittal sections were prepared from the embryos collected at multiple stages from timed mating. The sections were immunostained using an anti-ARF-BP1 antibody (Bethyl), which recognizes the middle portion of ARF-BP1 protein. As expected, ARF-BP1 could be detected in nucleus and cytoplasm in the cells from wild-type embryos (Fig. 2G). In contrast, almost all of the cells in arf-bp1 knock-out embryos had lost the staining of ARF-BP1 by E13.5, indicating sufficient depletion of ARF-BP1 protein in the cells from arf-bp1 knock-out embryos (Fig. 2J). Because ARF-BP1 has been shown to regulate p53 stability (11), sections from arf-bp1 knock-out embryos were immunostained using anti-p53 antibody (Novocastra). Indeed, staining of p53 was detected in neural cells at E13.5 (Fig. 2K), compared with those of the control embryos (Fig. 2H), demonstrating that ablation of ARF-BP1 resulted in accumulation of p53 protein. In addition, Cleaved Caspase3 staining was also readily detected in these cells in arf-bp1 knock-out embryos (Fig. 2L) compared with those of the control embryos (Fig. 2J), suggesting that accumulation of p53 led to activation of apoptosis. Together, these data suggest that the embryonic lethality of arf-bp1 knock-out mice was, at least, in part, due to p53 activation in the absence of functional ARF-BP1.

Ablation of ARF-BP1 in Pancreatic β-Cells in mice Resulted in Progressive Hyperglycemia and Development of Diabetes—Although the above data indeed validate the role of ARF-BP1 in p53 regulation in vivo, with very limited ARF-BP1-null cells/tissues, the physiological role of ARF-BP1 in p53 regulation could not be determined. The difficulty in studying these arf-bp1 knock-out mice has amplified the importance of generating additional ARF-BP1 mutant mice in different organs to facilitate the analysis of the biological phenotypes in adult tissues.

We chose to delete arf-bp1 in pancreatic β-cells because of the minimal effect on embryonic development by disruption of β-cell functions and the significant expression of ARF-BP1 in β-cells (Fig. 3A). Thus, we generated conditional arf-bp1 mutant mice with specific deletion of arf-bp1 in pancreatic β-cells, mediated by RIP-cre (24, 25). The arf-bp1fl/+/Y/RIP-cre mice were born close to Mendelian ratio and appeared normal; in particular, they had similar body weight compared with the control mice at least up to 6 months of age (supplemental Fig. 1). To determine whether arf-bp1 was efficiently ablated in β-cells, sections of pancreas from 1-month-old mice were immunostained using antibodies against ARF-BP1, insulin, and glucagon. ARF-BP1 staining was present in nucleus and cytoplasm of the β-cells of the control mice (Fig. 3A). Some of the β-cells showed no staining of ARF-BP1, presumably due to lack of expression of ARF-BP1 in proliferating cells, as observed previously (26). This is consistent with the fact that arf-bp1 conventional knock-out embryos died at late gestation stage.

### Table 1

| Stage (dpc) | Genotype | Offspring |
|------------|----------|-----------|
|            | +/+ , +/Y | +/−       | −/−       |
| Expected ratio | 2 | 1 | 1 |
| 11.5 | 2 | 2 | 4 |
| 12.5 | 21(1) | 10 | 12 |
| 13.5 | 33(1) | 12 | 17 |
| 14.5 | 12 | 6 | 4 |
| 16.5 | 18(1) | 4(1) | 8 |
| 17.5 | 5 | 2(1) | 2 |
| Total embryos | 91 | 36 | 47 |
| Offspring | 47 | 8 | 0 |

* Days postcoitus.

*The number of abnormal embryos is indicated in parentheses.

*All embryos had hemorrhage and were dead.

### FIGURE 2

**Mouse Conditional Knock-out of arf-bp1**

**A–F**, representative wild-type embryos (arf-bp1fl+/fl) (A–C) and arf-bp1 knock-out embryos (arf-bp1fl/fl) (D–F) from days E12.5, E14.5, and E16.5. G–L, sections of E13.5 embryos of wild-type embryos (G–I) and arf-bp1 knock-out embryos (J–L) immunostained using antibodies against ARF-BP1 (G and J), p53 (H and K), and Cleaved Caspase3 (I and L).
indicating critical functions of ARF-BP1 in postmitotic cells. In contrast, there was no staining for ARF-BP1 in the \( \beta \)-cells in the islets of \( \text{arf-bp1}^{FL/Y} / \text{RIP-cre} \) mice (Fig. 3Avi), demonstrating that ARF-BP1 was sufficiently depleted in the \( \beta \)-cells due to RIP-cre-mediated \( \text{arf-bp1} \) deletion. Consistent with the specificity of the RIP-cre expression, there was no difference of ARF-BP1 staining in the \( \alpha \)-cells of the islets and the surrounding acinar cells of the pancreas between \( \text{arf-bp1}^{FL/Y} / \text{RIP-cre} \) mice and the control mice (Fig. 3Aiv versus i). Interestingly, there was a similar staining for insulin in the \( \beta \)-cells (located in the core of the islet) (Fig. 3Av) in the absence of ARF-BP1 in \( \text{arf-bp1}^{FL/Y} / \text{RIP-cre} \) mice compared with that of the control mice (Fig. 3Avii). There was no significant difference for glucagon staining (located in the periphery of the islet) between the control and the \( \text{arf-bp1}^{FL/Y} / \text{RIP-cre} \) mice (Fig. 3A, iii versus vi).
To determine the long-term effects of arf-bp1 deletion on the β-cell functions, the blood glucose was monitored periodically. In addition, the proficiency of insulin response was determined by GTT at 3 and 6 months of age. Consistent with the insulin staining in islets from young mice, both fasting and fed glucose levels of arf-bp1FL/Y/RIP-cre mice showed similar levels during the first 2 months, compared with those of the control arf-bp1FL/Y mice (Fig. 3, B and C). However, as arf-bp1FL/Y/RIP-cre mice aged, blood glucose levels became increasingly higher than those of the control mice (Fig. 3, B and C). For the fed blood glucose levels (Fig. 3C), in particular, the average fed blood glucose level was 209 mg/dl for arf-bp1FL/Y/RIP-cre mice versus 167 mg/dl for the control mice at 3 months old; the differences increased to 308 versus 208 mg/dl at 6 months old and 373 versus 204 mg/dl at 9 months old (Fig. 3C). Consistently, fed insulin levels were decreased significantly in arf-bp1FL/Y/RIP-cre mice at 6 and 9 months old of age compared with those of the control mice (Fig. 3E). There were also significant lower fasting insulin levels for arf-bp1FL/Y/RIP-cre mice than the control mice at 9 months old (Fig. 3D). Furthermore, the insulin deficiency was confirmed by GTT. As shown in Fig. 3F, the glucose levels were significantly higher along the time course after glucose injection in arf-bp1FL/Y/RIP-cre mice than those of the control mice at 3

**FIGURE 4. Loss of pancreatic β-cells in aged arf-bp1FL/Y/RIP-cre mice.** A, representative 9-month-old control mouse (left) and arf-bp1FL/Y/RIP-cre mouse (right). Arrows indicate the bladders in the control and arf-bp1FL/Y/RIP-cre mice, and arrowheads indicate intestine in the control and arf-bp1FL/Y/RIP-cre mice. B, immunostaining of pancreases from the control (i–vi) and from the arf-bp1FL/Y/RIP-cre mice (vii–xii). i, iv, vii, and x, 1-month-old mice; ii, v, viii, and xi, 6-month-old mice; and iii, vi, ix, and xii, 9-month-old mice. Antibodies against insulin (i–iii and vii–ix) and glucagon (iv–vi and x–xii) were used. Dotted circles in ix outline the islets.
months old. The differences in GTT became even bigger for 6-month-old mice, suggesting further decline of insulin response in aged \textit{arf-bp1 FL/Y}/RIP-cre mice (Fig. 3G). Moreover, many of the \textit{arf-bp1 FL/Y}/RIP-cre mice (Fig. 4A, right) displayed typical signs for diabetes, such as excessive urination due to high blood glucose, and upon dissection, greatly enlarged bladder (Fig. 4A, arrows), and excess food in the intestine (Fig. 4A, arrowheads). Many of the mice had fed blood glucose levels higher than 600 mg/dl (limit of the glucometer). More than half of the mice died at 1 year of age, due to severe diabetic symptoms (see Fig. 6A, red line).

\textbf{Insulin Deficiency in \textit{arf-bp1 FL/Y}/RIP-cre Mice Was Caused by Loss of Pancreatic \textit{\beta}-Cells}—To determine the cause of the hyperglycemia and insulin deficiency, sections of pancreas from \textit{arf-bp1 FL/Y}/RIP-cre mice were collected and immunostained for insulin (\textit{\beta}-cells) and glucagon (\textit{\alpha}-cells). At 1 month old, the islets from \textit{arf-bp1 FL/Y}/RIP-cre mice had normal appearances of round shape, with center-located \textit{\beta}-cells and periphery-located \textit{\alpha}-cells (Fig. 4B, \textit{vii} and \textit{x} versus \textit{i} and \textit{iv}, respectively). At 6 months old, the morphology of islets in \textit{arf-bp1 FL/Y}/RIP-cre mice became increasingly irregular with frequent infiltration of \textit{\alpha}-cells in the middle of the islets (Fig. 4B,

\hspace{1cm}FIGURE 5. \textbf{Partial rescue of the hyperglycemia condition in \textit{arf-bp1 FL/Y}/RIP-cre mice by concomitant deletion of p53.} \textbf{A}, Western blot of the whole cell extracts from \textit{arf-bp1 FL/Y} MEFs infected with control adenovirus Ad-GFP (lane 1) or with Ad-Cre (lane 2) using antibodies against ARF-BP1, p53, p21, BAX, and \textit{\beta}-actin. \textbf{B} and \textbf{C}, fasting (\textbf{B}), and fed (\textbf{C}) blood glucose levels determined for control mice (blue bars), \textit{arf-bp1 FL/Y}/RIP-cre mice (red bars), and \textit{arf-bp1 FL/Y}/p53 FL/FL RIP-cre mice (green bars) 3, 6, and 9 months old. \textbf{D}, fed blood insulin levels determined for control mice (blue bars), \textit{arf-bp1 FL/Y}/RIP-cre mice (red bars), and \textit{arf-bp1 FL/Y}/p53 FL/FL RIP-cre mice (green bars) 6 and 9 months of age. \textbf{E}, GTT for control mice (blue line), \textit{arf-bp1 FL/Y}/RIP-cre mice (red line), and \textit{arf-bp1 FL/Y}/p53 FL/FL RIP-cre mice (green line) 6 months of age. Data are presented as mean \pm S.D. (error bars). Statistical significance was assessed using Student’s \textit{t} test (*, \textit{p} < 0.05; **, \textit{p} < 0.001).
Diabetic Conditions of arf-bp1FL/Y; RIP-cre mice Were Significantly Rescued by Concomitant p53 Ablation—Our previous study showed that ARF-BP1 plays an important role in downregulation of p53 because RNAi-mediated knockdown of ARF-BP1 increases the basal levels (~3-fold) of p53 proteins and enhances p53-mediated functions in human cell lines (11). Thus, it is very likely that the cause for the aging-dependent decrease of β-cell population in arf-bp1FL/Y; RIP-cre mice is the high basal levels of p53 in these ARF-BP1-depleted β-cells and that these cells are more sensitive to normal physiological stress. To confirm the role of ARF-BP1 in regulating p53 function, we decided to delete arf-bp1 in arf-bp1FL/Y MEFs infected with Cre-expressing adenovirus (Ad-cre), which allowed detailed analysis of the effects on p53 function by arf-bp1 inactivation. As shown in Fig. 5A, whereas ARF-BP1 was expressed as a protein of about 500 kDa in wild-type cells (lane 1), no ARF-BP1 protein was detected in arf-bp1FL/Y cells after Ad-cre virus infection (Fig. 5A, lane 2), demonstrating depletion of ARF-BP1 protein after deletion of arf-bp1. Significantly, p53 was accumulated in arf-bp1 knockout cells, which led to activation of its targets p21 and apoptotic gene BAX (Fig. 5A, lane 2 versus lane 1). These results showed that deletion of arf-bp1 indeed led to stabilization and activation of p53.

To determine further whether loss of β-cells in arf-bp1FL/Y RIP-cre mice was due to activation of p53 under physiological settings, we generated arf-bp1FL/Y;p53FL/F RIP-cre double knock-out mice, in which both arf-bp1 and p53 were deleted in β-cells. Dramatically, the hyperglycemia conditions in arf-bp1FL/Y; RIP-cre mice were alleviated in arf-bp1FL/Y;p53FL/F RIP-cre mice. Both fasting (Fig. 5B, green bar) and fed (Fig. 5C, green bar) blood glucose levels were lowered in arf-bp1FL/Y; p53FL/F RIP-cre mice compared with those of the arf-bp1FL/Y; RIP-cre mice (Fig. 5, B and C, red bar). Consistently, insulin levels in arf-bp1FL/Y;p53FL/F RIP-cre mice (Fig. 5D, green bar) were higher than that of arf-bp1FL/Y; RIP-cre mice (Fig. 5D, red bar). Furthermore, GTT using 6-month-old mice revealed significant improvement of insulin response in arf-bp1FL/Y;p53FL/F RIP-cre mice (Fig. 5E, green line), as the glucose level at the end of the assay for arf-bp1FL/Y; p53FL/F RIP-cre mice was nearly the same as that of the control mice (Fig. 5E, green line versus blue line). In contrast, the glucose levels for arf-bp1FL/Y; RIP-cre mice were significantly higher than that of the control mice at the end of the assay (Fig. 5E, red line). Notably, the double knock-out mice survived much longer than the arf-bp1FL/Y; RIP-cre mice, due to overall improvement of the diabetic condition (Fig. 6A, green line).

Furthermore, histology analysis of pancreases revealed similar architecture of islets and the abundance of β-cells in arf-bp1FL/Y;p53FL/F RIP-cre mice (Fig. 6H) compared with the arf-bp1FL/Y;p53FL/F 9-month-old mice (Fig. 6F), whereas the α-cells maintained their peripheral localization (Fig. 6, I versus G). These histological features represented significant improvement of arf-bp1FL/Y;p53FL/F RIP-cre mice compared with arf-bp1FL/Y; RIP-cre mice, which showed dramatic reduction of insulin staining and disorganized islets (Fig. 6, D and E). Insulin and glucagon staining of 9-month-old arf-bp1FL/Y mice was included for comparison (Fig. 6, B and C, respectively). Together, these results suggested that the diabetic phenotype in arf-bp1FL/Y; RIP-cre mice was significantly rescued by p53 gene ablation.

**DISCUSSION**

This study demonstrated that arf-bp1FL/Y; RIP-cre mice developed an age-dependent diabetic phenotype, due to loss of
Mouse Conditional Knock-out of arf-bp1

β-cells. Evidence suggested that activation of p53 caused reduction of β-cell population in arf-bp1FL/−/RIP-cre mice, as concomitant knock-out of p53 significantly restored β-cell population and rescued the diabetic phenotype in arf-bp1FL/−/RIP-cre mice. These findings are consistent with the function of ARF-BP1 in ubiquitination and stability of p53, which is also validated by the study of arf-bp1 knock-out embryos and arf-bp1 knock-out MEFs. Interestingly, recent studies revealed that up-regulation of p53 contributes to β-cell apoptosis and diabetes, highlighting the importance of controlling p53 activation in β-cells (27–30). In addition, several studies have shown an age-dependent decline of β-cell regeneration and functions, due to increase of expression of ARF (31–33). It is intriguing whether ARF regulates β-cell functions through its ability to inhibit ARF-BP1. Although several E3 ubiquitin ligases have been identified to regulate p53 (34, 35), ARF-BP1 is the first E3 ubiquitin ligase of p53, other than Mdm2, shown to regulate p53 functions under physiological settings. Moreover, although our results demonstrate that p53 is regulated by ARF-BP1 in vivo, deregulation of other substrates of ARF-BP1 may also contribute to the phenotype of ARF-BP1 mutant mice, which clearly needs further elucidation in the future.

The p53 protein is well established as a major tumor suppressor in almost every type of human cancers because of its crucial functions in coordinating cellular responses to genotoxic stress and its irreplaceable roles in suppressing tumorigenesis. p53 is a short-lived protein whose activity is maintained at low levels by both Mdm2-dependent and Mdm2-independent mechanisms in normal cells. The cellular functions of p53 are rapidly activated in response to stress, and tight regulation of p53 is essential for its effect on tumorigenesis as well as maintaining normal cell growth. Importantly, restoration of p53 activity remains an important goal in the quest for more effective cancer therapies. Indeed, Nutlin-3, a small molecule inhibitor of Mdm2, is able to activate p53 and exhibits antitumor efficacy in cancer cells that express wild-type p53 (36). Notably, in some cases the activity of p53 can be dangerous for the organism. Thus, p53-dependent apoptosis induced in normal tissues during chemotherapeutic and radiotherapy can cause severe side effects of antitumor therapy and, therefore, limits its efficiency. Our study showed that p53 activation in arf-bp1 mutant mice is the major cause of severe diabetic symptoms and shortened life span. More importantly, the diabetic phenotype of these mice was largely reversed by p53 ablation, and the life span of the mice was also extended. Further studies are required to examine whether p53 is indeed overactivated in diabetic patients. If so, it will be interesting to test whether pifithrin-α, a previously unidentified small molecule inhibitor of p53 (37, 38), is beneficial in the treatment of diabetes. In this regard, it is also interesting to know whether small molecule activators of SirT1, a deacetylase of p53 (39, 40), can improve glucose homeostasis and insulin sensitivity, partially through modulating p53 function.

REFERENCES
1. Zhang, Y., and Lu, H. (2009) Signaling to p53: ribosomal proteins find their way. Cancer Cell 16, 369–377
2. Kruse, J. P., and Gu, W. (2009) Modes of p53 regulation. Cell 137, 609–622
3. Vousten, K. H., and Prives, C. (2009) Blinded by the light: the growing complexity of p53. Cell 137, 413–431
4. Qian, Y., and Chen, X. (2010) Tumor suppression by p53: making cells senescent. Histol. Histopathol. 25, 515–526
5. Marine, J. C., and Lozano, G. (2010) Mdm2-mediated ubiquitination: p53 and beyond. Cell Death Differ. 17, 93–102
6. Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995) Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature 378, 206–208
7. Montes de Oca Luna, R., Wagner, D. S., and Lozano, G. (1995) Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 378, 203–206
8. Ringshausen, I., O’Shea, C. C., Finch, A. J., Swigart, L. B., and Evan, G. I. (2006) Mdm2 is critically and continuously required to suppress lethal p53 activity in vivo. Cancer Cell 10, 501–514
9. Dornan, D., Wertz, I., Shimizu, H., Arnot, D., Frantzt, G. D., Dowd, P., O’Rourke, K., Koeppen, H., and Dixit, V. M. (2004) The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature 429, 86–92
10. Leng, R. P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, I. M., Lozano, G., Hakem, R., and Benichimol, S. (2003) Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. Cell 112, 779–791
11. Chen, D., Kon, N., Li, M., Zhang, W., Qin, J., and Gu, W. (2005) ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. Cell 121, 1071–1083
12. Zhang, X., Berger, F. G., Yang, J., and Lu, X. (2011) USP4 inhibits p53 through deubiquitinating and stabilizing ARF-BP1. EMBO J. 30, 2177–2189
13. Eisen, C. M., and Lozano, G. (2009) p53 and MDM2: antagonists or partners in crime? Cancer Cell 15, 161–162
14. Zhang, Y. (2004) The ARF-B23 connection: implications for growth control and cancer treatment. Cell Cycle 3, 259–262
15. Sherr, C. J. (2006) Divorcing ARF and p53: an unsettled case. Nat. Rev. Cancer 6, 663–673
16. Hall, J. R., Kow, E., Nevis, K. R., Lu, C. K., Luce, K. S., Zhong, Q., and Cook, J. G. (2007) Cdc6 stability is regulated by the Huw1 ubiquitin ligase after DNA damage. Mol. Biol. Cell 18, 3340–3350
17. Yin, L., Joshi, S., Wu, N., Tong, X., and Lazar, M. A. (2010) E3 ligases Arf-bp1 and Pam mediate lithium-stimulated degradation of the circadian heme receptor Rev-erba. Proc. Natl. Acad. Sci. U.S.A. 107, 11614–11619
18. Liu, Z., Miao, D., Xia, Q., Hermo, L., and Wing, S. S. (2007) Regulated expression of the ubiquitin protein ligase, E3(histone)/LASU1/Mule/ARF-BP1/HUWE1, during spermatogenesis. Dev. Dyn. 236, 2889–2898
19. Liu, P., Jenkins, N. A., and Copeland, N. G. (2003) A highly efficient recombineering-based method for generating conditional knockout mutations. Genome Res. 13, 476–484
20. Chen, Z., Trotman, L. C., Shaffer, D., Lin, H. K., Dotan, Z. A., Niki, M., Koutcher, J. A., Scher, H. I., Ludwig, T., Gerald, W., Gordon-Cord, C., and Pandolfi, P. P. (2005) Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature 436, 725–730
21. Herrera, P. L. (2000) Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development 127, 2317–2322
22. Xuan, S., Kitamura, T., Nakae, J., Politi, K., Kido, Y., Fisher, P. E., Morroni, M., Cinti, S., White, M. F., Herrera, P. L., Accili, D., and Eistriatidis, A. (2002) Defective insulin secretion in pancreatic β-cells lacking type 1 IGF receptor. J. Clin. Invest. 110, 1011–1019
23. Zhao, X., D’Arca, D., Lim, W. K., Brahmachary, M., Carro, M. S., Ludwig, T., Cardo, C. C., Guillenot, F., Aldape, K., Califano, A., Iavarone, A., and Lasorella, A. (2009) The N-Myc-DLL3 cascade is suppressed by the ubiqui-
uitin ligase Huw1 to inhibit proliferation and promote neurogenesis in the developing brain. *Dev Cell* 17, 210–221

27. Gurzov, E. N., Germano, C. M., Cunha, D. A., Ortis, F., Vanderwinden, J. M., Marchetti, P., Zhang, L., and Eizirik, D. L. (2010) p53 up-regulated modulator of apoptosis (PUMA) activation contributes to pancreatic β-cell apoptosis induced by proinflammatory cytokines and endoplasmic reticulum stress. *J. Biol. Chem.* 285, 19910–19920

28. Armata, H. L., Golebiowski, D., Jung, D. Y., Ko, H. J., Kim, J. K., and Sluss, H. K. (2010) Requirement of the ATM/p53 tumor suppressor pathway for glucose homeostasis. *Mol. Cell. Biol.* 30, 5787–5794

29. Hinault, C., Kawamori, D., Liew, C. W., Maier, B., Hu, J., Keller, S. R., Mirmira, R. G., Scrable, H., and Kulkarni, R. N. (2011) 40 Isoform of p53 controls proliferation and glucose homeostasis in mice. *Diabetes* 60, 1210–1222

30. Bitti, M. L., Saccucci, P., Capasso, F., Piccinini, S., Angelini, F., Rapini, N., Porcaro, M., Arcano, S., Petrelli, A., Del Duca, E., Bottini, E., and Gloria-Bottini, F. (2011) Genotypes of p53 codon 72 correlate with age at onset of type 1 diabetes in a sex-specific manner. *J. Pediatr. Endocrinol. Metab.* 24, 437–439

31. Chen, H., Gu, X., Su, I. H., Bottino, R., Contreras, J. L., Tarakhovsky, A., and Kim, S. K. (2009) Polycomb protein Ezh2 regulates pancreatic β-cell Ink4a/Arf expression and regeneration in diabetes mellitus. *Genes Dev.* 23, 975–985

32. Krishnamurthy, J., Ramsey, M. R., Ligon, K. L., Torrice, C., Koh, A., Bonner-Weir, S., and Sharpless, N. E. (2006) p16Ink4a induces an age-dependent decline in islet regenerative potential. *Nature* 443, 453–457

33. Tschen, S. I., Dhawan, S., Gurlo, T., and Bhushan, A. (2009) Age-dependent decline in β-cell proliferation restricts the capacity of β-cell regeneration in mice. *Diabetes* 58, 1312–1320

34. Migliorini, D., Bogaerts, S., Defever, D., Vyas, R., Denecker, G., Radaelli, E., Zwolinska, A., Depaepe, V., Hochepeed, T., Skarnes, W. C., and Marine, J. C. (2011) COP1 constitutively regulates c-Jun protein stability and functions as a tumor suppressor in mice. *J. Clin. Invest.* 121, 1329–1343

35. Vitari, A. C., Leong, K. G., Newton, K., Yee, C., O’Rourke, K., Liu, J., Phu, L., Vij, R., Ferrando, R., Couto, S. S., Mohan, S., Pandita, A., Hongo, J. A., Arnott, D., Wertz, I. E., Gao, W. Q., French, D. M., and Dixit, V. M. (2011) COP1 is a tumor suppressor that causes degradation of ETS transcription factors. *Nature* 474, 403–406

36. Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N., and Liu, E. A. (2004) *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 303, 844–848

37. Komarov, P. G., Komarova, E. A., Kondratov, R. V., Christov-Tselkov, K., Coon, J. S., Chernov, M. V., and Gudkov, A. V. (1999) A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* 285, 1733–1737

38. Gudkov, A. V., and Komarova, E. A. (2005) Prospective therapeutic applications of p53 inhibitors. *Biochem. Biophys. Res. Commun.* 331, 726–736

39. Michan, S., and Sinclair, D. (2007) Sirtuins in mammals: insights into their biological function. *Biochem. J.* 404, 1–13

40. Milne, J. C., Lambert, P. D., Schenk, S., Carney, D. P., Smith, J. J., Gagne, D. J., Jin, L., Boss, O., Perni, R. B., Vu, C. B., Bernis, J. E., Xie, R., Disch, J. S., Ng, P. Y., Nunes, J., Lynch, A. V., Yang, H., Galonetz, H., Israelian, K., Choy, W., Illland, A., Lavu, S., Medvedik, O., Sinclair, D. A., Olefsky, J. M., Jirousek, M. R., Elliott, P. J., and Westphal, C. H. (2007) Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature* 450, 712–716