Supplementary Information for:

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Supplementary Material and Methods

Cell lines and primary cells

The following human cancer cell lines were obtained from American Type Culture Collection (ATCC): RT-4 (HTB-2), UMUC3 (CRL-1749), T24 (HTB-4), PC3 (CRL-1435), J82 (HTB-1), DU145 (HTB-81), Hela (CCL-2), and TCCSUP (HTB-5). Mouse embryonic fibroblasts (MEFs) were made from the following mutant mouse alleles: a conditional allele for *p53* (*p53*$^{f/f}$; FVB; 129SJv) having loxP sites flanking exons 2 and 10 (1), obtained from the NCI Mouse Models of Human Cancer Consortium (MMHCC) repository; a conditional allele for *Pten* (*Pten*$^{f/f;}$ C57Bl6; 129SVJ) having loxP sites flanking exon 5 (2), also from the NCI-MMHCC repository; and a conditional allele for *p19*^{Arf} (*Arf*$^{f/f}$; C57Bl6; 129SJv) having loxP sites flanking exon 1b of *Cdkn2a* (3), obtained from Dr. Charles Sherr. Mice were mated to obtain the relevant combinations of mutant alleles and MEFs were isolated from 13.5 *dpc* mouse embryos following standard procedures. Gene deletion of loxP sites was induced by infecting MEFs with a Cre recombinase-expressing Adenovirus (Adeno-Cre; University of Iowa Vector Core Facility) at high titer of 10^8 TU/mL with 8 μg/ml polybrene. The consequences of gene deletion for expression of the relevant proteins and mRNA were confirmed Western blot analyses and real-time PCR, respectively.

Tissue microarray

The bladder cancer and prostate cancer tissue microarrays (TMAs) used in this study are described in Supplementary Table S1. The bladder cancer TMA was comprised of surgical specimens from 89 patients who underwent radical cystectomy at Memorial Sloan-Kettering Cancer Center; median follow up was 22 months (range 2 to 200). The prostate cancer TMA was generated from surgical specimens from 128

patients who underwent radical prostatectomy at Columbia Medical Center; median follow up was 101 months (range 2 to 222). TMAs were constructed having 3 cores from each patient. Consecutive sections from the TMAs were immunostained with anti-ARF or anti-p16 antibodies or analyzed by H&E (to verify pathology). The antibodies used in this study are described in Supplementary Table S3, following the procedure described in (4). Immunostaining of each core was scored by estimating the percentage of immune-reactive tumor cells as well as the intensity of the staining using an ordinal scale (undetectable = 0, weak = 1, strong = 2). If different staining intensities were found in a core, the most intense staining (>25% of cells) was used for final analysis. Each case had 3 cores and mean score of 3 cores were calculated and recorded as a score for each case. Statistical analyses were done using the two-sided student t-test. Survival analysis was conducted by the log rank test. All calculations were performed using Prism (GraphPad Software, Inc. La Jolla, CA).

Cell culture analyses

A REGγ cDNA was cloned into pcDNA3.1+ as a fusion protein with an HA epitope. A $p14^{ARF}$ or p16 cDNA was subcloned into the pMXs IRES-RFP (pMXs-IR) vector, which also expresses RFP to enrich for infected cells by cell sorting. Cells were infected with the ARF-expressing retrovirus (or empty vector) along with 8 μg/ml polybrene. siRNA were purchased from Sigma-Aldrich and transfected using Lipofectamine RNAiMAX (Invitrogen); sequences are provided in Supplementary Table S2. Where indicated, cells were treated with: cycloheximide in dimethyl sulfoxide (DMSO) from Sigma-Aldrich (Catalog #C4859) at 50 μg/ml; bortezomib from LC labs (Catalog #B-1408) dissolved at 5 mM in DMSO and used at 5 μM; and/or ginkgolic acid from Calbiochem (Catalog #345887) dissolved in DMSO to make a working stock of 50

mM and used at 5 μM unless otherwise indicated; and/or Leptomycin B in ethanol from LC Laboratories (Catalog #L-6100) at 1mM (540 μg/ml) and used at 50 ng/ml unless otherwise indicated.

Western blot assays were done using total protein lysates extracted in RIPA buffer (0.1% SDS, 1.0% Deoxycolate-Sodium salt, 1.0% Triton X-100, 0.15 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA) with fresh protease inhibitor (Roche) and phosphatase inhibitor (Sigma-Aldrich). For immunoprecipitation, cells were lysed in 50 mM Tris-HCl, pH7.4, 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40 containing protease inhibitor cocktail (Roche) and the lysates incubated at 4˚C with primary antibody followed by precipitation with protein G or A. A summary of antibodies used in this study is provided in Supplementary Table S3. Signal intensity of Western blot assays was quantified using ImageJ and half-lives were estimated by drawing approximate reduction curves. For analyses of mRNA levels, real-time PCR analysis was performed using a Quantitech SYBR Green PCR (Qiagen). Sequences of oligos used in this study are provided in Supplementary Table S2. A Pair Wise Fixed Reallocation Randomization Test using the Relative Expression Software Tool (REST) (Qiagen) was used to test the significance of the expression ratios of transcripts.

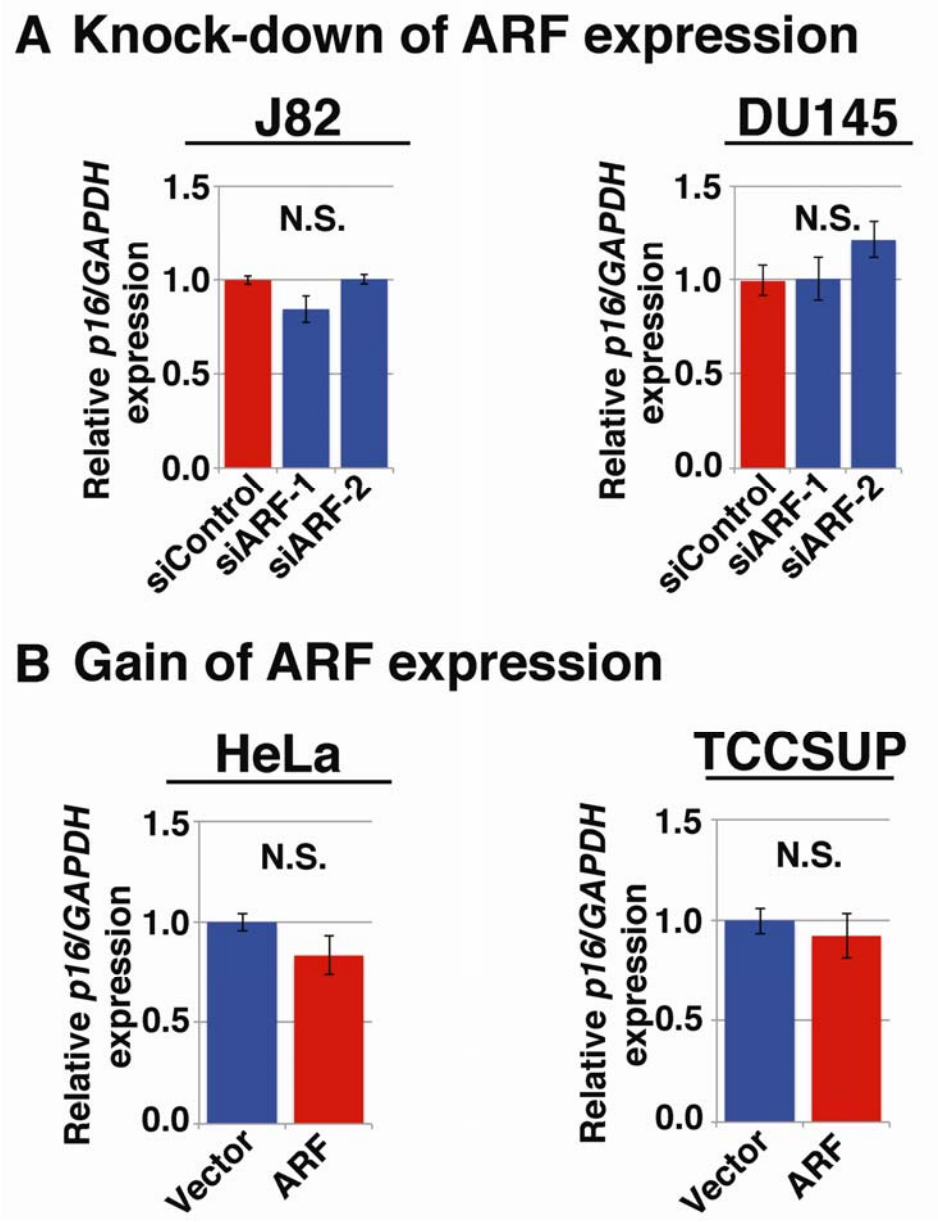
Immunofluorescence analyses were done as described previously (5). Briefly Arf(+) and Arf(—) MEFs were seeded on 1-well BD Falcon™ CultureSlide and transfected with the HA tagged REGγ plasmid. Cells were fixed in 4% PFA in PBS with 1% sucrose and permeabilized by incubation in an isotonic solution, 0.5% Triton X-100 (10% sucrose, 50 mM NaCl, 6 mM $MgCl_2$, 20 mM HEPES [pH 7.2], 0.5% Triton X-100). After blocking with 1% BSA (bovine serum albumin) in PBS, cells were incubated for 1.5 hours at room temperature with primary antibody (HA antibody). Following incubation with primary antibody, samples were washed in PBS containing 0.1% Tween 20 followed

by incubation for 1 hour with TOPRO 3 and AlexaFluor 555 secondary antibodies (Invitrogen). Immunofluorescence staining was visualized using a Leica TCS SP5 inverted confocal microscope equipped with argon/krypton and helium/neon lasers capable of excitation wavelengths 488, 555, and 642 nm. Details of antibodies are provided in Supplementary Table S3.

Supplementary Reference

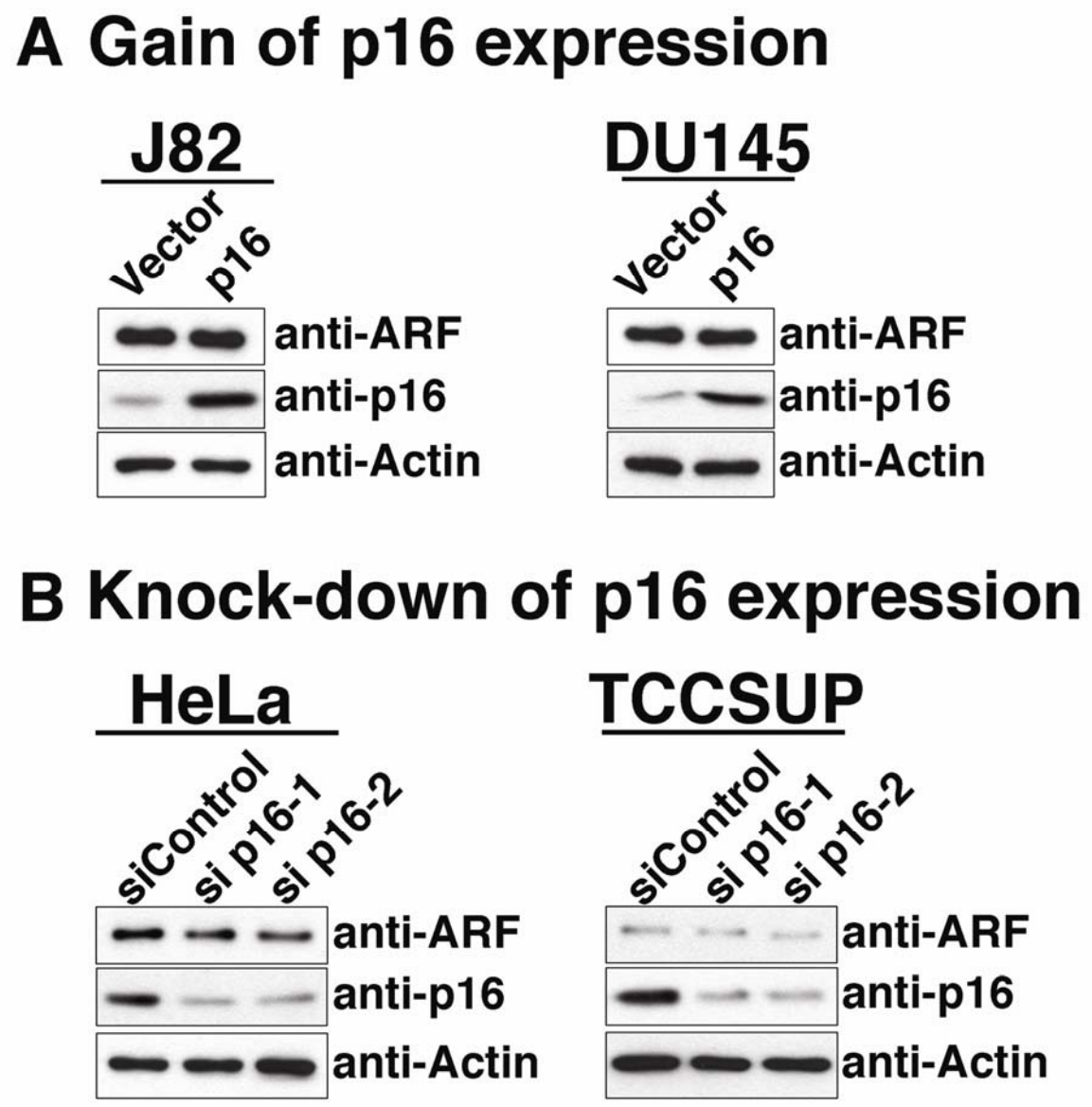
1. Jonkers J, Meuwissen R, van der Gulden H, Peterse H, van der Valk M, Berns A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet. 2001;29:418-25.
2. Lesche R, Groszer M, Gao J, Wang Y, Messing A, Sun H, et al. Cre/loxP-mediated inactivation of the murine Pten tumor suppressor gene. Genesis. 2002;32:148-9.
3. Gromley A, Churchman ML, Zindy F, Sherr CJ. Transient expression of the Arf tumor suppressor during male germ cell and eye development in Arf-Cre reporter mice. Proceedings of the National Academy of Sciences of the United States of America. 2009;106:6285-90.
4. Kinkade CW, Castillo-Martin M, Puzio-Kuter A, Yan J, Foster TH, Gao H, et al. Targeting AKT/mTOR and ERK MAPK signaling inhibits hormone-refractory prostate cancer in a preclinical mouse model. The Journal of clinical investigation. 2008;118:3051-64.
5. Wang J, Kumar RM, Biggs VJ, Lee H, Chen Y, Kagey MH, et al. The Msx1 Homeoprotein Recruits Polycomb to the Nuclear Periphery during Development. Developmental cell. 2011;21:575-88.

Supplementary Figure S1



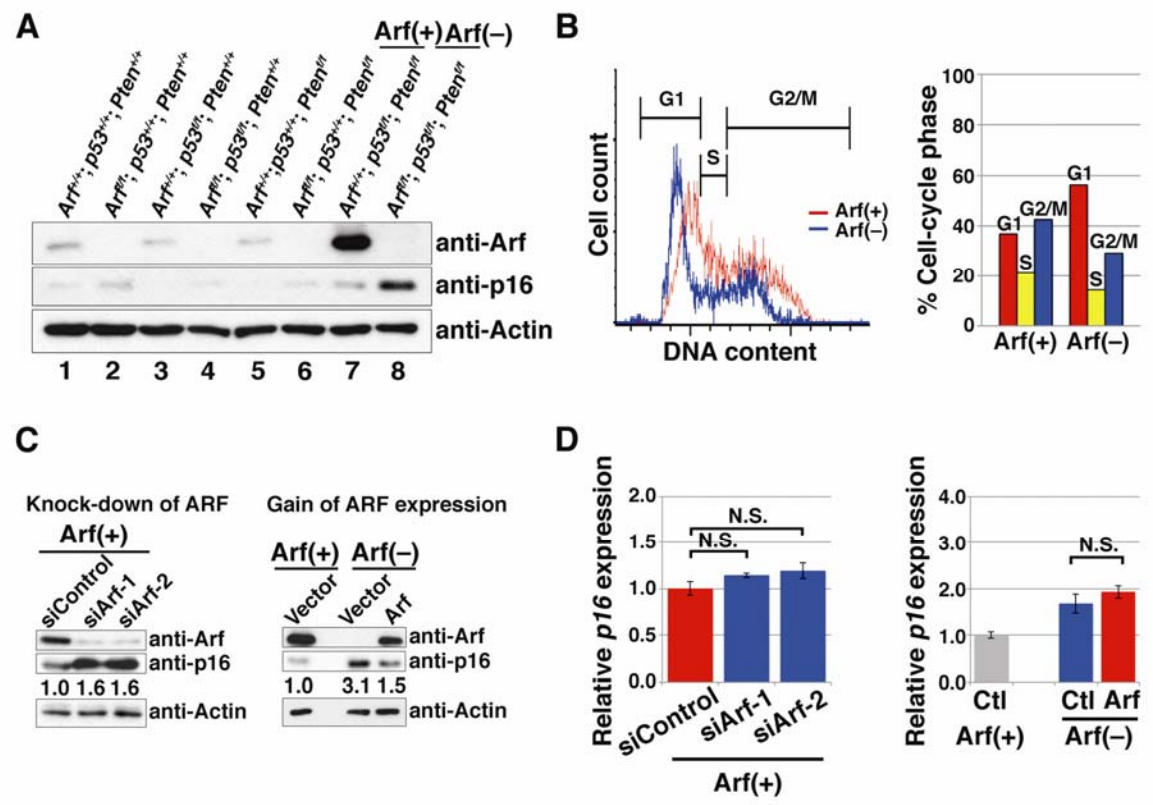
Supplementary Figure S1. ARF expression does not affect *p16* mRNA levels in human cancer cell lines. Quantitative PCR analyses of *p16* mRNA levels for the indicated cells following knock-down of ARF in J82 or DU45 cells or following expression of exogenous ARF in HeLa or TCCSUP cells. (See Figure 1 for details).

Supplementary Figure S2



Supplementary Figure S2. p16 does not affect ARF protein expression levels in human cancer cell lines. (A) Consequences of expressing exogenous p16 in J82 and DU145 cells following transfection with a p16 cDNA (or the empty vector as a control). (B) Consequences of p16 knock-down in HeLa and TCCSUP cells using two independent p16 siRNA (or a scrambled siRNA as a control).

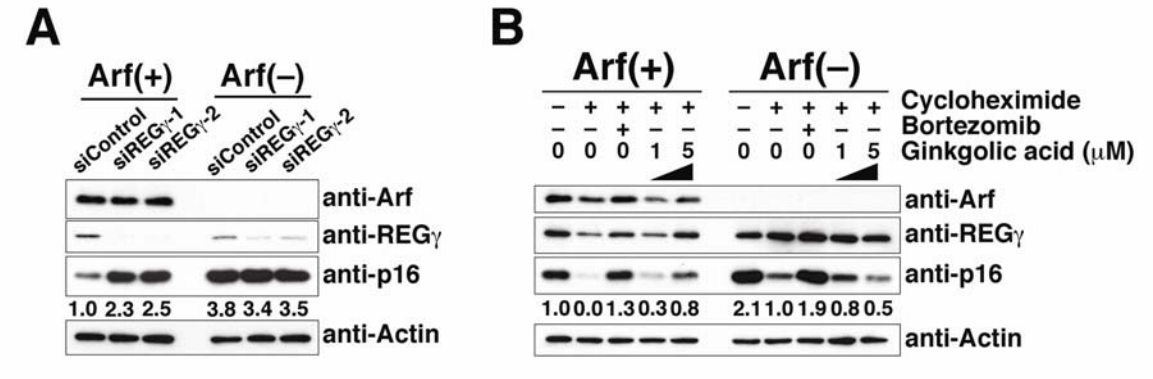
Supplementary Figure S3



Supplementary Figure S3. Characterization of Arf-positive and Arf-null mouse embryonic fibroblasts (MEFs). (A) Protein expression levels of Arf and p16 protein in MEFs of the indicated genotypes, shown by Western blotting. Note that Arf and p16 protein levels are inversely correlated in the MEF cells. However, Arf is robustly expressed in the *Arf*$^{+/+}$*; p53*$^{f/f}$*; Pten*$^{f/f}$ MEFs (which we refer to as Arf(+)) while p16 is robustly expressed in the *Arf*$^{f/f}$*; p53*$^{f/f}$*; Pten*$^{f/f}$ MEFs (which we refer to Arf(—)). (B) Cell cycle analyses comparing the Arf(+) and Arf(—) MEFs show that the Arf(—) MEFs are enriched in G1 phase. *(Left)* Histogram from cell-cycle analyses using fluorescence-activated cell sorting (FACS). (*Right*) Distribution of cell-cycle phase in Arf(+) and Arf(—) MEFs. (C) Gain or loss of expression of Arf affects p16 protein levels. (*Left)* Arf knock-down in Arf(+) MEFs increases p16 protein levels. Western blot analyses of Arf(+) MEFs transfected with two different Arf siRNA (or a control siRNA). *(Right)* Expression of

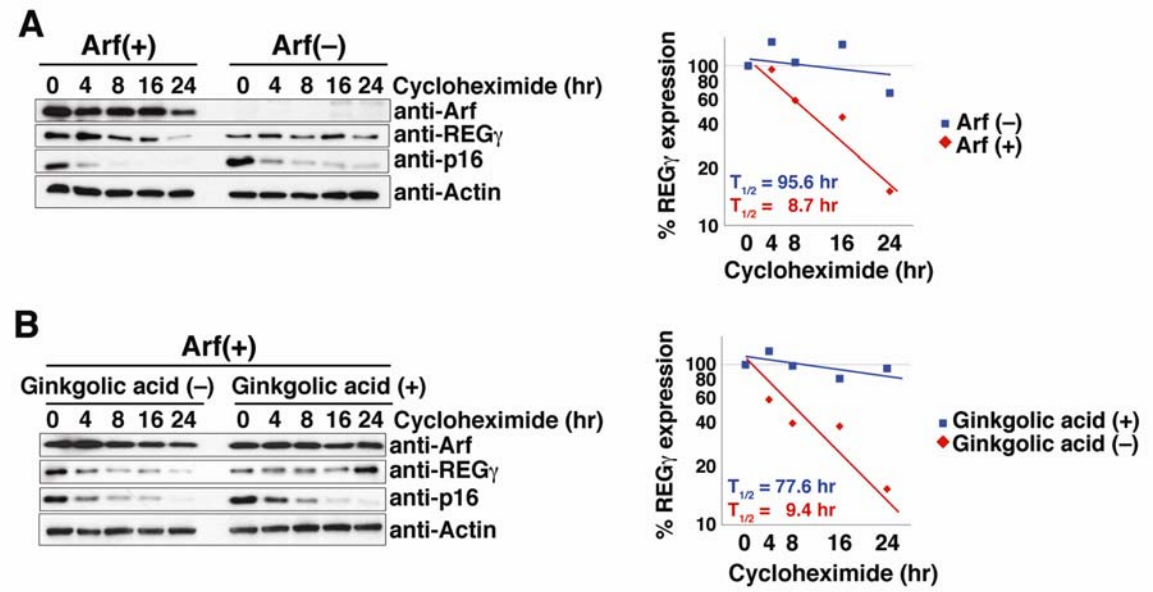
exogenous Arf reduces p16 protein levels in Arf(—) MEFs. Western blot analyses of Arf(—) MEFs transfected with an Arf cDNA (or control vector). (D) Gain or loss of expression of Arf does not affect *p16* mRNA levels. (*Left)* Arf-positive MEFs were transfected with Arf siRNA or the control siRNA and quantitative PCR was done to examine p16 mRNA expression. *(Right)* Arf-null MEFs were transfected with exogenous Arf or the empty vector and quantitative PCR was done to examine p16 mRNA expression. In *C* the relative expression levels of p16 are indicated as determined using ImageJ software.

Supplementary Figure S4



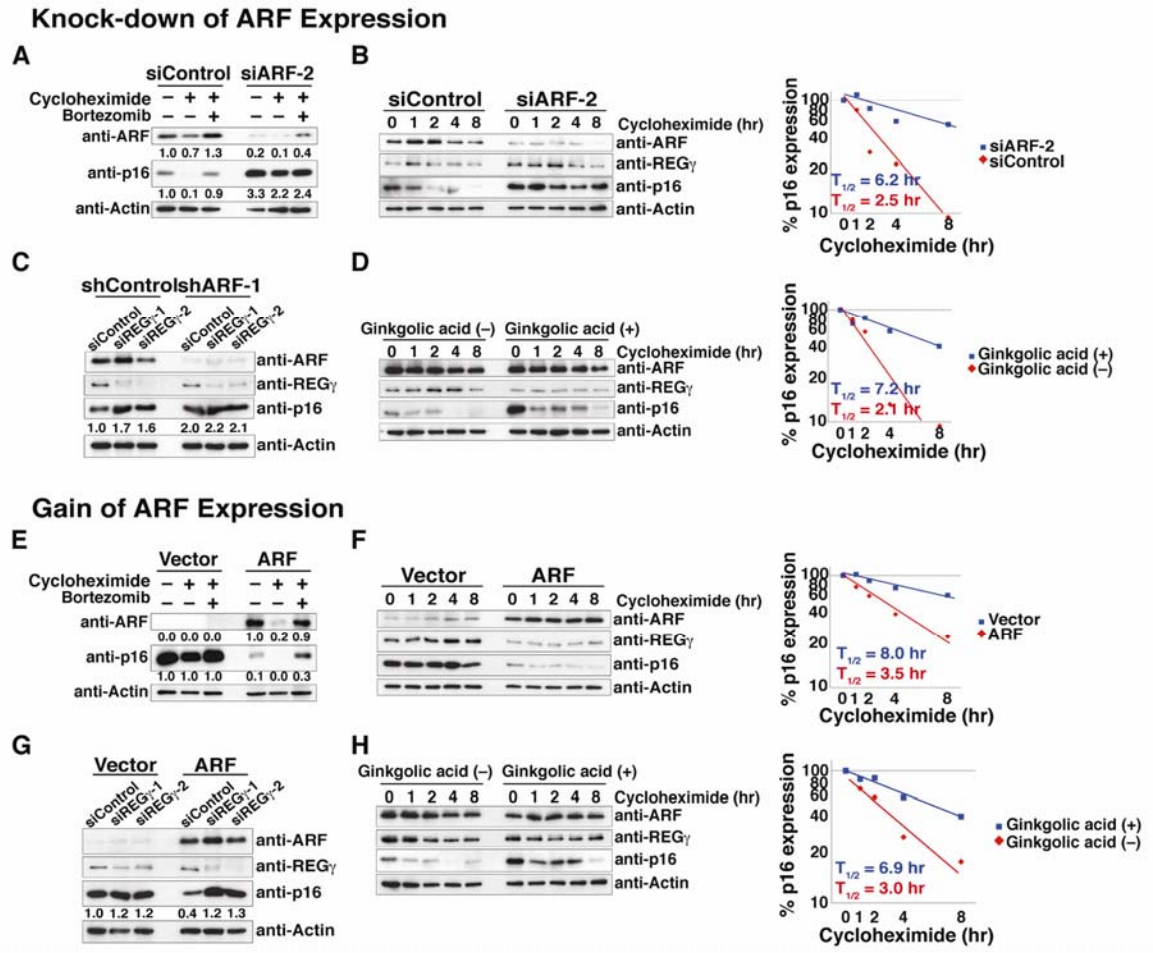
Supplementary Figure S4. Arf regulates the stability of p16 protein via REGγ-dependent proteasome degradation in MEFs. (A) Knock-down of REGγ abrogates the Arf-dependent suppression of p16 protein levels. Arf(+) and Arf(—) MEFs were treated with two independent REGγ siRNA (or a scrambled siRNA as a control) and proteins analyzed by Western blotting. (B) Arf-mediated destablization of p16 protein levels can be counteracted by the SUMOylation inhibitor, ginkgolic acid. Arf(+) and Arf(—) MEFs were untreated or treated with cycloheximide (50 μg/ml) with or without bortezomib (5 μM) in the presence or absence of ginkgolic acid (1 or 5 μM, as indicated) and analyzed by Western blot analyses. The relative expression levels of p16 are indicated as determined using ImageJ software.

Supplementary Figure S5



Supplementary Figure S5. REGγ is degraded in an Arf-dependent and SUMOylation-dependent manner. (A) Arf(+) and Arf(—) MEFs were treated with cycloheximide (50 μg/ml) for indicated time. (B) Arf(+) MEFs were treated (+) or not (—) with ginkgolic acid (5 μM) for 4 hr prior to addition of cycloheximide (50 μg/ml) for indicated time. Western blot was done to evaluate the expression levels of the indicated proteins. In (A and B) panels on the left show protein levels by western blotting and panels on the right show expression of REGγ versus time and half-life ($T_{1/2}$) calculated based on approximation curves. The relative expression levels of REGγ are indicated as determined using ImageJ software.

Supplementary Figure S6



Supplementary Figure S6. ARF regulates the stability of p16 protein via REGγ-dependent proteasome degradation in human cancer cells. Analyses were done: *(in A-D)* in J82 cells (which express ARF, see Fig. 1A) following knock-down of ARF using two alternative siRNA (or a control), and *(in E-H)* in HeLa cells (which lack ARF, see Fig. 1A) following transfection with exogenous ARF cDNA (or the control vector). (A, E) ARF-mediated destablilization of p16 protein is counteracted by proteasome inhibitor. J82 cells (with control or ARF siRNA) or HeLa cells (with exogenous ARF or the control vector) were untreated or treated cycloheximide (50 μg/ml) with or without bortezomib (5 μM) followed by Western blot analyses. (B, F) ARF regulates the stability of p16 protein. J82 cells (with control or ARF siRNA) or HeLa cells (with exogenous ARF or the control

vector) were treated with cycloheximide (50 μg/ml) for the indicated time in hours. *(Left)* Western blot analyses showing relative protein expression levels. *(Right)* Relative change in p16 expression as a function of time showing the half-life ($T_{1/2}$) was calculated from approximation curves. (C, G) Knock-down of REGγ abrogates the ARF-dependent suppression of p16 protein levels. J82 cells (with control or ARF siRNA) or HeLa cells (with exogenous ARF or the control vector) were treated with two independent REGγ siRNA (or a scrambled siRNA as a control) and proteins analyzed by Western blotting. (D, H) Inhibition of SUMOylation stabilizes p16 expression. J82 cells or HeLa cells (with exogenous ARF) were treated or untreated with ginkgolic acid (5 μM) for 4 hr followed by treatment with cycloheximide (50 μg/ml) for the indicated time in hours. *(Left)* Western blot analyses showing relative protein expression levels. *(Right)* Relative change in p16 expression as a function of time showing the half-life ($T_{1/2}$) was calculated from approximation curves. The relative expression levels of p16 are indicated as determined using ImageJ software.

Supplementary Table S1: Description of bladder and prostate TMAs

A

Bladder cancer cohort (TMA of 89 patients)	
Age (median ± S.D.)	71 ± 12
Gender (%)	
Male	59 (66)
Female	30 (34)
p-T stage (%)	
T1 or less	14 (16)
T2	13 (15)
T3	45 (51)
T4	17 (19)
p-N stage (%)	
N0	52 (58)
N1-2	21 (24)
Unknown	16 (18)
Status of ARF (%)	
Negative	59 (55)
Positive	40 (45)
Status of p16 (%)	
Negative	33 (37)
Positive	56 (63)

B

Prostate cancer cohort (TMA of 128 patients)	
Age (median ± S.D.)	61 ± 7
PSA (median ± S.D.)	8 ± 17
p-T stage (%)	
T2	53 (41)
T3	63 (50)
T4	6 (5)
Unknown	6 (5)
p-N stage (%)	
N0	110 (86)
N1	5 (4)
Unknown	13 (10)
Gleason score	
5-6	35 (27)
7	59 (46)
8-10	28 (22)
Unknown	6 (5)
Status of ARF (%)	
Negative	70 (55)
Positive	57 (45)
Status of p16 (%)	
Negative	74 (58)
Positive	53 (42)

Supplementary Table S2: List of DNA/RNA oligos used in this study.		
Purpose and name	**Sequence**	
Quantitative PCR	**Forward**	**Reverse**
***p16* (human)**	GCCCAACGCACCGAATAGT	CACCAGCGTGTCCAGGAAG
***GAPDH* (human)**	GGTGAAGGTCGGAGTCAACG	AGGGATCTCGCTCCTGGAAG
***p16* (mouse)**	AGGAGAGCCATCTGGAGCAG	CTGCTCCAGATGGCTCTCCT
***Gapdh* (mouse)**	CTAGAGAGCTGACAGTGGGTAT	AGACGACCAATGCGTCCAAA
siRNA	**Sense**	**Antisense**
p14ARF siRNA#1 (human)	CUCGUGCUGAUGCUACUGAtt	UCAGUAGCAUCAGCACGAGgg
p14ARF siRNA#2 (human)	GGUCCCAGUCUGCAGUUAAtt	UUAACUGCAGACUGGGACCca
p16 siRNA#1 (human)	GCUAAGUGCUCGGAGUUAAtt	UUAACUCCGAGCACUUAGCga
p16 siRNA#2 (human)	GCAUGGAGCCUUCGGCUGAtt	UCAGCCGAAGGCUCCAUGCga
p19Arf siRNA#1 (mouse)	GGCCGCCGCUGAGGGAGUAtt	UACUCCCUCAGCGGCGGCCtt
p19Arf siRNA#2 (mouse)	GAGCUGCGCUCUGGCUUUCtt	GAAAGCCAGAGCGCAGCUCtt
REGγ siRNA#1 (human)	GAAGGAAAGUGCUAGGUGUtt	ACACCUAGCACUUUCCUUCtt
REGγ siRNA#2 (human)	CUCAUCAUAUCAGAGCUGAtt	UCAGCUCUGAUAUGAUGAGtt
REGγ siRNA#1 (mouse)	GGAAACAGUUGCUGAACUAtt	UAGUUCAGCAACUGUUUCCtt
REGγ siRNA#2 (mouse)	GACAUUGCCUUGGUUUGUUtt	AACAAACCAAGGCAAUGUCtt

Supplementary Table S3: List of antibodies used in this study.								
Antigen Name	**Company**	**Catolog #**	**Type**	**Species[1]**	**Use and dilution[2]**			
					Western	**Immuno-histochemistry**	**Immuno-precipitation**	**Immuno-fluorescence**
β-actin (ACTB)	Cell Signaling	#4970 (clone #13E5)	Rabbit mAb	Mouse/Human	1:1000	N/A	N/A	N/A
p16	Santa Cruz	#sc-1207	Rabbit pAb	Mouse/Human	1:500	1:100	N/A	N/A
p16	Calbiochem	#NA29 (clone #DCS-50.1/H4)	Mouse mAb	Human	1:1000	N/A	N/A	N/A
p16	mtm laboratories AG	CINtec® Histology V-Kit	Mouse mAb	Human	N/A	Ready to use	N/A	N/A
$p19^{Arf}$	Abcam	#ab80	Rabbit pAb	Mouse	1:500	1:125	1:50	1:50
$p14^{ARF}$	Cell Signaling	#2407 (clone #4C6/4)	Mouse mAb	Human	1:100	1:100	N/A	N/A
REGγ	ZYMED	#38-3800	Rabbit pAb	Mouse/Human	1:1000	N/A	N/A	N/A
FLAG	SIGMA	F1804	Mouse mAb	Mouse	1:1000	N/A	N/A	N/A
HA	Roche	#11-867-423001	Rat mAb	Mouse	1:1000	N/A	1:50	1:500
Anti-rabbit secondary	Amersham/GE	ECL Rabbit IgG, HRP-Linked Whole Ab (NA934)	Donkey	Mouse/Human	1:25000	N/A	N/A	N/A
Anti-mouse secondary	Amersham/GE	ECL Rabbit IgG, HRP-Linked Whole Ab (NA931)	Sheep	Mouse/Human	1:25000	N/A	N/A	N/A
Biotinylated anti-rabbit	Vector laboratories	Biotinylated anti-rabbit IgG (H+L) (BA-1000)	Goat	Mouse/Human	N/A	1:300	N/A	N/A
Anti-mouse secondary	Biocare Medical	Mouse on Mouse, HRP polymer (MM510H)	Goat	Mouse/Human	N/A	1:300	N/A	N/A
Anti-rat secondary	Invitrogen	Alexa Fluor® 555 Goat Anti-Rat IgG (H+L) (A21434)	Goat	Mouse	N/A	N/A	N/A	1:500
TOPRO3	Invitrogen	T3605	N/A	Mouse	N/A	N/A	N/A	1:1000
Notes: (1) Indicates the experimental species for which the antibody was used in the current study. (2) N/A not applicable (did not use in this capacity).								