Post-translational Down-regulation of Melanoma Antigen-A11 (MAGE-A11) by Human p14-ARF Tumor Suppressor*

John T. Minges[‡], Gail Grossman[‡], Ping Zhang[§], Tal Kafri[§¶‖], and Elizabeth M. Wilson[‡¶‡‡1]**

From the [‡]Laboratories for Reproductive Biology, Department of Pediatrics, [§]Lentivirus Core Facility, [¶]Lineberger Comprehensive Cancer Center, [‖]Gene Therapy Center, and Departments of [**]Microbiology and Immunology and [‡‡]Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

Background: Melanoma antigen-A11 (MAGE-A11) is a primate-specific steroid receptor coregulator and proto-oncogene expressed at increased levels in castration-resistant prostate cancer.

Results: Human p14-ARF tumor suppressor promotes the proteasomal degradation of MAGE-A11 independent of ubiquitination.

Conclusion: MAGE-A11 is post-translationally down-regulated by the p14-ARF tumor suppressor.

Significance: Increased levels of MAGE-A11 associated with low p14-ARF promote the development of castration-resistant prostate cancer.

X-linked primate-specific melanoma antigen-A11 (MAGE-A11) is a human androgen receptor (AR) coactivator and proto-oncogene expressed at low levels in normal human reproductive tract tissues and at higher levels in castration-resistant prostate cancer where it is required for androgen-dependent cell growth. In this report, we show that MAGE-A11 is targeted for degradation by human p14-ARF, a tumor suppressor expressed from an alternative reading frame of the p16 cyclin-dependent kinase inhibitor *INK4a/ARF* gene. MAGE-A11 degradation by the proteasome was mediated by an interaction with p14-ARF and was independent of lysine ubiquitination. A dose-dependent inverse relationship between MAGE-A11 and p14-ARF correlated with p14-ARF inhibition of the MAGE-A11-induced increase in androgen-dependent AR transcriptional activity and constitutive activity of a splice variant-like AR. Reciprocal stabilization between MAGE-A11 and AR did not protect against degradation promoted by p14-ARF. p14-ARF prevented MAGE-A11 interaction with the E2F1 oncoprotein and inhibited the MAGE-A11-induced increase in E2F1 transcriptional activity. Post-translational down-regulation of MAGE-A11 promoted by p14-ARF was independent of HDM2, the human homologue of mouse double minute 2, an E3 ubiquitin ligase inhibited by p14-ARF. However, MAGE-A11 had a stabilizing effect on HDM2 in the absence or presence of p14-ARF and cooperated with HDM2 to increase E2F1 transcriptional activity in the absence of p14-ARF. We conclude that degradation of MAGE-A11 promoted by the human p14-ARF tumor suppressor contributes to low levels of MAGE-A11 in nontransformed cells and that higher levels of MAGE-A11 associated with low p14-ARF increase AR and E2F1 transcriptional activity and promote the development of castration-resistant prostate cancer.

Prostate cancer growth and progression depend on androgen receptor (AR)[2] transcriptional signaling, which is increased by melanoma antigen-A11 (MAGE-A11), an AR coregulator. MAGE-A11 is a cancer-testis antigen that resides predominantly in the nucleus at low levels in normal human reproductive tract tissues and at higher levels in castration-resistant prostate cancer (1–6). Although named initially for its identification in melanoma (7, 8), MAGE-A11 is a coregulator of human AR and progesterone receptor-B (1, 6, 9). MAGE-A11 increases AR transcriptional activity by binding an extended AR NH$_2$-terminal FXXLF motif region independent of AR binding the active androgens, testosterone or dihydrotestosterone (DHT) (1). MAGE-A11 stabilizes the unliganded AR and facilitates recruitment of p300 and p160 transcriptional coactivators during androgen-dependent AR transactivation (1, 10, 11).

The *MAGE-A11* gene at the Xq28 locus of the MAGE gene family on the human X chromosome evolved within the primate lineage by gene duplication and retrotransposition (12, 13). The functional dependence on MAGE-A11 for increased human AR transcriptional activity is supported by the coevolution of X-linked human *MAGE-A11* and X-linked human AR NH$_2$-terminal sequence flanking the FXXLF motif that mediates AR interaction with MAGE-A11 and the androgen-dependent AR NH$_2$- and COOH-terminal interaction (14, 15). Rat and mouse AR Ala[33] evolved to Val[33] in human AR NH$_2$-terminal sequence [23]FQNLFQSVREV[33], a sequence change required for human AR to interact with MAGE-A11 (15). However, neither mouse nor rat AR Ala[33] or human AR Val[33] is required for the FXXLF motif-mediated androgen-dependent AR NH$_2$- and COOH-terminal interaction. These findings suggest that the increase in human AR transcriptional activity resulting from the coactivator activity of MAGE-A11 evolved more recently among primates than evolution of the androgen-dependent AR NH$_2$- and COOH-terminal interaction.

[2] The abbreviations used are: AR, androgen receptor; MAGE-A11, melanoma-antigen-A11; DHT, dihydrotestosterone; ARF, p14-alternative reading frame.

In addition to AR coregulator activity, MAGE-A11 has functions that directly impact the cell cycle and require complex regulatory control. MAGE-A11 interacts with and increases the transcriptional activity of E2F1, one of the most important transcription factors and oncoproteins that promotes unscheduled entry into S phase of the cell cycle (5). The increase in E2F1 activity by MAGE-A11 is similar to the effects of E1A, an adenovirus early protein that transforms cells (16). The ability of MAGE-A11 to increase AR and E2F1 transcriptional activity suggests that MAGE-A11 links AR to cell cycle progression and supports the concept that cancer cells continue to grow through the increased expression and activity of oncoproteins such as E2F1 and MAGE-A11.

The unrestrained growth of cancer cells also depends on deregulation of tumor suppressors, such as human p14-ARF (ARF, alternative reading frame) and p53, proteins that protect normal cells from tumorigenesis by arresting the cell cycle or inducing apoptosis. ARF derives from the *INK4a/ARF* locus in an alternative reading frame by alternate promoter usage and splicing that differs from the p16 cyclin-dependent kinase inhibitor that is more often mutated in cancer (17–21). Human p14-ARF shares only 50% homology with the p19-ARF mouse homologue (22), which indicates that the *INK4a/ARF* gene continued to evolve late within the mammalian lineage similar to the *MAGE-A11* gene and AR NH_2-terminal F*XX*LF motif flanking sequence required to interact with MAGE-A11. A primary function of ARF is to bind and inhibit HDM2 (human homologue of mouse double minute 2), an E3 ligase that ubiquitinates and promotes the degradation of p53 (23–27). Inhibition of HDM2 E3 ubiquitin ligase activity by ARF increases p53 levels, which induces cell cycle arrest or apoptosis. The inhibitory activity of ARF that promotes p53 accumulation requires a unique ARF exon 1B region (22). Loss of ARF or p53 activity in cancer cells allows cellular proto-oncogenes such as E2F1 and MAGE-A11 to promote uncontrolled cancer cell growth and the development of castration-resistant prostate cancer.

In this report we provide evidence that human ARF promotes the proteasomal degradation of MAGE-A11 independent of HDM2 E3 ubiquitin ligase or lysine ubiquitination. ARF inhibited the ability of MAGE-A11 to stabilize and stimulate AR or E2F1 transcriptional activity but did not overcome a stabilizing effect of MAGE-A11 on HDM2. The findings suggest that ARF maintains normal low levels of MAGE-A11 to limit cell proliferation and preserve the nontransformed phenotype, whereas low levels of ARF in prostate cancer cells contribute to higher levels of MAGE-A11 that promote the development of castration-resistant prostate cancer.

Experimental Procedures

DNA Vectors—Mammalian expression vectors include full-length 1–429-amino acid human MAGE-A11 expressed in pSG5-MAGE, FLAG-MAGE (1), and VP-MAGE (1, 9, 11); full-length 1–919-amino acid human AR in pCMV-AR (28), pCMV-AR-(1–660) NH_2-terminal and DNA-binding domain fragment (29), and pCMV-AR-(1–660)-L26A,F27A F*XX*LF motif mutant (14); and pSG5-HA-p300 (10). pCMV-E2F1 and pBabe-puro were provided by Y. Xiong (University of North Carolina at Chapel Hill) (5, 30). FLAG-E2F1 was provided by

E. S. Robertson (University of Pennsylvania) (31). pCI-neo-HA-ARF and pBabe-puro-hARF coding for human ARF, pCMVβ-HA2-HDM2, and pCMVβ-HDM2 were provided by W. G. Yarbrough (Yale University). Luciferase reporter vectors included PSA-Enh-Luc from M. Carey (University of California Los Angeles) (10, 32), E2F1-Luc from J. R. Nevins (Duke University) (10, 33) and 5XGAL4Luc3 from D. P. McDonnell (Duke University) (34, 35). GAL-ARF was prepared by PCR-amplifying pCI-neo-HA-ARF using oligonucleotide primers to omit the HA tag and produce a fragment cloned into EcoR1 and BamHI sites of GAL-O. FLAG-ARF was prepared by digesting GAL-ARF with EcoRI and BamHI and inserting the fragment into pCMV-FLAG-b.

Expression and RNA Analysis—Cell media contained penicillin, streptomycin, and 2 mM L-glutamine (Gibco, Life Technologies) as follows: human LAPC-4 cells, RPMI 1640 with 10% FBS and 1 nM R1881 synthetic androgen agonist; 22Rv1 cells, RPMI 1640 with 10% FBS; LNCaP cells, RPMI 1640 with 2% FBS; human HeLa cervical cancer cells, minimal essential medium with 10% FBS; CV1 and COS1 monkey kidney cells, DMEM with 10% bovine calf serum and 20 mM Hepes, pH 7.2; PC-3 and DU145 cells, DMEM with 10% FBS; CWR-R1, DMEM with additives (2).

Transcription assays were performed in 12-well plates containing 1 ml medium/well plated with 5×10^4 HeLa cells/well or 4×10^4 CV1 cells/well and transfected using 0.6 μl of X-tremeGENE DNA transfection reagent (Roche) and 50 μl medium/well. AR transcriptional activity was measured in CV1 cells using 0.25 μg of PSA-Enh-Luc, 25 ng of pCMV-AR, 10 ng of pCMV-AR-(1–660), and/or 25 ng of pCI-neo-HA-ARF. Endogenous E2F1 transcriptional activity was measured in HeLa cells using 0.1 μg of E2F1-Luc, 0.1 μg of pSG5-MAGE, and increasing amounts of pCI-neo-HA-ARF. Mammalian two-hybrid assays were performed in HeLa cells using 0.1 μg of 5XGAL4Luc3, 50 ng of GAL-ARF, 50 ng of VP16 empty vector, or wild-type or mutant VP-MAGE. The day after transfection, cells were incubated for 24 h in serum-free medium with or without 10 nM DHT. Luciferase activity measurements represent the mean and S.E. of at least three independent experiments.

Immunoblot analysis was performed on endogenous protein in cancer cells or expressed protein in CV1 or COS1 cells. CV1 cells (2.3×10^5 cells/6-cm dish) were transfected in duplicate 6-cm dishes with 3 ml of medium using 4 μl of X-tremeGENE reagent, up to 2 μg of DNA/dish and 80 μl of medium/6-cm dish, or 5×10^5 CV1 cells/10-cm dish with 6 ml of medium using 8 μl of X-tremeGENE reagent, 160 μl of medium, and up to 4 μg DNA/dish. COS1 cells (6.2×10^5 cells/6-cm dish or 2×10^6 cells/10-cm dish) were transfected using DEAE dextran (36, 37).

Control and human p14-ARF retrovirus derived from pBabe-puro or pBabe-puro-hARF retroviral vectors were prepared using HEK293T cells. Retrovirus in 0.25 ml of HEK293T cell media was added to 2×10^6 LAPC-4 cells/well or 6.2×10^5 HeLa cells/well in 6-well plates containing 2 ml of medium/well and incubated for 48 h at 37 °C. Confluent cells were selected over 9 days by initially passaging 2 wells into a 10-cm dish. After cell attachment, 3 μg/ml puromycin was included in the

medium that was exchanged every 2 or 3 days. LAPC-4 or HeLa cells not treated with retrovirus were killed within 5 days of treatment with 3 μg/ml puromycin. Quantitative RT-PCR of RNA extracted using TRIzol from LAPC-4 and HeLa pBabe-control and pBabe-ARF-expressing cells was performed using sequence specific primers for MAGE-A11 and peptidylprolyl isomerase A control as described (38).

Lentivirus shRNA—Endogenous ARF expression was inhibited using a pLK01-modified lentiviral vector that contains an RNA polymerase III-shRNA expression cassette and a puromycin resistance gene under the control of the phosphoglycerate kinase promoter. ARF shRNA targeted the 5′-CTCGTGCT-GATGCTACTGAgg-3′ sense sequence in the unique ARF exon 1B (antisense sequence ccTCAGTAGCATCAGCAC-GAG-3′). Lentiviral vectors carrying nonspecific shRNA or empty vector with no shRNA served as negative controls. Vector particles ($\sim$10^6 IU/ml) were generated by a three-plasmid transfection in HEK293 cells (39). HeLa cells (3×10^5 cells/well in 6-well plates) were transduced using 0.1 or 0.25 ml of vector-containing conditioned media for 48 h at 37 °C and selected for puromycin resistance over several days in the presence of 3 μg/ml puromycin.

Immunochemistry—Immunoblot analysis was performed on cell extracts prepared in lysis buffer that contained 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 2 mM EDTA, 0.05 mM NaF, 2 mM sodium vanadate, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM PMSF, and complete protease inhibitors (Roche Applied Science). Immunoprecipitation experiments were performed by extracting cells in lysis buffer that contained 1% Triton X-100, 0.5% deoxycholate, 0.15 M NaCl, 0.05 M NaF, 1 mM EDTA, 50 mM Tris-HCl, 1 mM DTT, 1 mM PMSF, and complete protease inhibitors and processed as described (5).

Immunoblots of 10 or 12% acrylamide gels containing SDS were probed using the following antibodies: MAGE1 or MAGE2 rabbit polyclonal antibody against baculovirus expressed FLAG-tagged human MAGE-A11 (0.5–10 μg/ml) (5); rabbit polyclonal anti-VP16 tag ab4809 antibody (1:500 dilution, Abcam); rabbit polyclonal human p14-ARF 10437 antibody provided by W. G. Yarbrough (1:200 dilution); rabbit polyclonal AR32 antibody against a human AR NH$_2$-terminal peptide (1–3 μg/ml) (40); rabbit polyclonal human p300 antibody sc-585 (1:200 dilution, Santa Cruz Biotechnology); rabbit polyclonal E2F1 antibody sc-193 (1:200 dilution, Santa Cruz Biotechnology); HA tag 12ca5 mouse monoclonal antibody (University of North Carolina Antibody Core Facility, 1–2 μg/ml); anti-FLAG M2 F3165 mouse monoclonal antibody (1:200 dilution, Sigma); anti-GAL4 DNA-binding domain rabbit polyclonal sc-577 antibody (1:500 dilution, Santa Cruz Biotechnology); mouse monoclonal p27^{Kip1} 610241 antibody (1:200 dilution, BD Biosciences); and β-actin ab6276 mouse antibody (1:5000 dilution, Abcam). Immunoblots were calibrated using dual color Precision Plus protein standards (Bio-Rad). Immunoreactivity was detected by chemiluminescence using SuperSignal West Dura extended duration substrate (Pierce). Protein bands were quantitated using ImageQuant TL Software (GE Healthcare Life Sciences).

Cell Growth Assays—LAPC-4 cell growth assays were performed using cells that stably express retrovirus derived from pBabe-puro control or pBabe-puro-hARF retroviral vectors. LAPC4 control and LAPC-4-ARF cells were plated in triplicate (4×10^5 cells/well) in 24-well plates with 0.5 ml of phenol red-free growth medium containing 10% charcoal-stripped serum (Atlanta Biologicals). The next day 0.1 ml of phenol red-free, serum-free medium without DHT was added or with DHT for a final concentration of 1 nM DHT. Cells were retreated with or without 1 nM DHT after 72 h on day 3. Plates were aspirated 24 h after addition of DHT and daily thereafter. Serum-free medium (0.2 ml) and 20 μl of Cell Counting Kit-8 reagent (Dojindo Laboratories) were added/well and incubated at 37 °C for 2.5 h. Optical absorbance was determined at 485 nm. Statistical significance was determined using two-way ANOVA.

Results

Interaction with Human ARF Promotes the Degradation of MAGE-A11—MAGE-A11 coregulator activity depends on interactions with the ligand-activated human AR and p300 transcriptional coactivator (1, 6, 10, 11). MAGE-A11 also interacts with and increases the transcriptional activity of the E2F1 oncoprotein that promotes cell cycle progression through interactions with p107 of the retinoblastoma family (5). Based on the ability of MAGE-A11 to stabilize and increase E2F1 transcriptional activity, and the ARF tumor suppressor to promote E2F1 degradation and inhibit E2F1 transcriptional activity (41, 42), we investigated whether MAGE-A11 is a direct target of human ARF or proteins regulated by ARF.

Interaction between MAGE-A11 and ARF was demonstrated by the coimmunoprecipitation of HA-ARF with FLAG-MAGE (Fig. 1A), and by MAGE-A11 coimmunoprecipitation with FLAG-ARF (Fig. 1B). There was no evidence for nonspecific association of ARF or MAGE-A11 with the FLAG antibody resin. However, expression of ARF decreased MAGE-A11 levels in cell extracts by 86% (Fig. 1A) or 44% (Fig. 1B) when normalized to the β-actin protein loading control.

An interaction between MAGE-A11 and ARF was also evident in a mammalian two-hybrid assay. Activity of a luciferase reporter gene linked to GAL4 DNA binding sites was increased by 6-fold with the coexpression of an ARF-GAL4 DNA-binding domain fusion protein and a full-length MAGE-A11-VP16 activation domain fusion protein (Fig. 1C). The similar activity induced by GAL-ARF in the presence of full-length 1–429-amino acid VP-MAGE or VP-MAGE-(112–429) suggested that ARF interacts with a COOH-terminal region of MAGE-A11. This was consistent with a weak two-hybrid interaction between GAL-ARF and VP-MAGE-(2–110) that contains only the MAGE-A11 NH$_2$-terminal region or between GAL-ARF and VP-MAGE-(112–362) that contained the central region of MAGE-A11. GAL-ARF, VP-MAGE, and VP-MAGE fragments expressed at similar levels, except for VP-MAGE-(2–110), which was expressed at a lower level (Fig. 1D). The results suggest an interaction between MAGE-A11 and ARF requires the COOH-terminal region of MAGE-A11.

The decline in MAGE-A11 associated with ARF expression (Fig. 1, A and B) suggested that ARF increases the degradation of MAGE-A11. This was supported by a dose-dependent
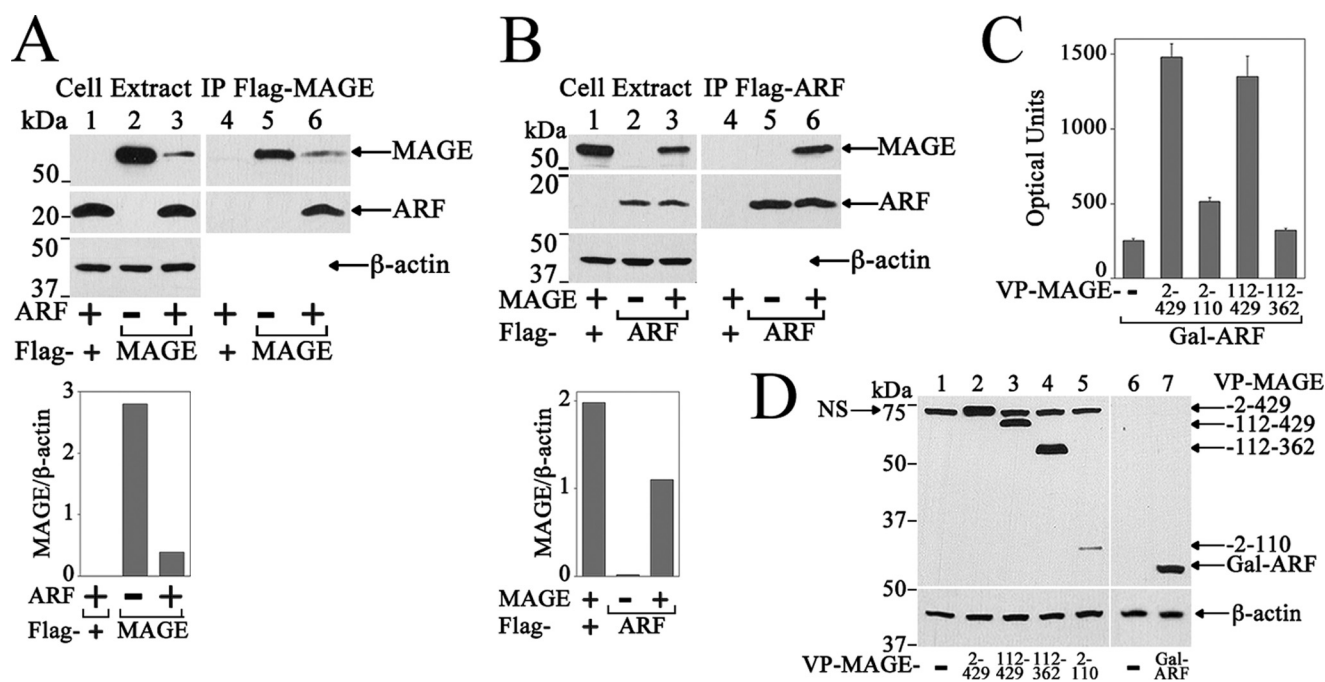
FIGURE 1. **MAGE-A11 interaction with ARF.** *A*, immunoprecipitation of ARF was performed in COS1 cells using 5 μg of pCMV-FLAG or 5 μg of pCMV-FLAG-MAGE with or without 6 μg of pCI-neo-HA-ARF/10-cm dish. Cell extracts (80 μg of protein/lane, *left panel*) and immunoprecipitates (*IP*, *right panel*) were probed using FLAG-M2, HA, and β-actin antibodies. MAGE-A11 *versus* β-actin band intensity in cell extracts is shown in the *lower panel*. *B*, immunoprecipitation of MAGE-A11 was performed in COS1 cells using 6 μg of pCMV-FLAG or 6 μg of FLAG-ARF with or without 3 μg of pSG5-MAGE/10-cm dish. Extracts (60 μg of protein/lane, *left panel*) and immunoprecipitates (*IP*, *right panel*) from cells treated with 5 ng/ml EGF for 24 h before harvest were probed using FLAG-M2, MAGE2, and β-actin antibodies. MAGE-A11 *versus* β-actin band intensity in cell extracts is shown in the *lower panel*. *C*, mammalian two-hybrid assay demonstrating MAGE-A11 interaction with ARF was performed in HeLa cells by expressing 0.1 μg of 5XGAL4Luc3 with 50 ng of GAL-ARF and 50 ng of pVP16 ($-$), full-length VP-MAGE-(2–429), or VP-MAGE-(2–110), 112–429, or 112–362. *D*, expression levels were compared by expressing per 6-cm COS1 cell dish 6 μg of VP16 empty vector (*lane 1*), VP-MAGE-(2–429), 112–429, 112–362 or 2–110 (*lanes 2–5*), GAL-O empty vector (*lane 6*), or GAL-ARF (*lane 7*). Cells were incubated with 1 μM MG132 for 24 h prior to harvest. Cell extracts (70 μg of protein/lane) were probed on an immunoblot using VP16 and GAL4 antibodies. A nonspecific (*NS*) VP16 antibody immunoreactive band of ~72 kDa migrated similar to VP-MAGE-(2–429).

inverse relationship between ARF and MAGE-A11 relative to β-actin (Fig. 2*A*). In contrast, there was a direct relationship between ARF and AR (Fig. 2*B*) that was lost with expression of MAGE-A11 (Fig. 2*C*). When expressed together, the levels of both AR and MAGE-A11 decreased with increasing ARF. The results suggest that the susceptibility of MAGE-A11 to degradation promoted by ARF was extended to AR through the interaction between AR and MAGE-A11.

Inhibition of protein synthesis using cycloheximide provided additional evidence that ARF increases the degradation rate of MAGE-A11 (Fig. 2*D*). The intracellular half-life of MAGE-A11 decreased from ~8 to 4 h with the expression of ARF.

The region in MAGE-A11 required for degradation promoted by ARF was investigated based on the similar susceptibility of MAGE-A11 (Fig. 2*A*) and a full-length GAL-MAGE fusion protein (Fig. 2*E*, *lanes 1* and *2*) to degradation promoted by ARF. The stability of GAL-MAGE-(2–121) or GAL-MAGE-(2–205) in the presence of ARF (Fig. 2*E*, *lanes 3–6*) provided further evidence that the MAGE-A11 COOH-terminal region is targeted for degradation by ARF. In contrast, GAL-MAGE-(112–429) and GAL-MAGE-(112–276), which share the central region of MAGE-A11, were down-regulated by ARF similar to full-length GAL-MAGE (Fig. 2*F*).

The results show that ARF promotes the degradation of MAGE-A11 independent of the MAGE-A11 NH-terminal region or AR. Increased degradation of AR promoted by ARF was evident only in the presence of MAGE-A11.

MAGE-A11 Degradation by the Proteasome Promoted by Human ARF Independent of Ubiquitination—Dependence on the proteasome and lysine ubiquitination for MAGE-A11 degradation promoted by ARF was investigated using chemical inhibitors and alanine substitution mutants. MAGE-A11 levels in the presence of ARF and normalized to β-actin increased 1.3-fold after incubation with 1 μM MG132 and 2.7-fold with 10 μM MG132 (Fig. 3*A*). A similar increase in MAGE-A11 was seen in the presence of ARF and 5 μM bortezomib, another proteasome inhibitor (43) (Fig. 3*B*). The results provided evidence that ARF promotes MAGE-A11 degradation by the proteasome.

The requirement for lysine ubiquitination in the proteasomal degradation of MAGE-A11 promoted by ARF was examined by first establishing a minimal region of MAGE-A11 that could be subjected to lysine mutagenesis. In agreement with results in Fig. 2 (*E* and *F*), the MAGE-(2–252) NH$_2$-terminal fragment did not interact with FLAG-ARF (Fig. 3*C*, *lanes 4* and *11*). This was in contrast to a strong interaction between ARF and MAGE-(112–429) and MAGE-(112–362) and a weaker interaction with MAGE-(112–276) (Fig. 3*C*, *lanes 5–7* and *12–14*).

Because MAGE-(112–276) interacted with ARF (Fig. 3*C*) and was down-regulated by ARF (Fig. 2*F*), we constructed MAGE-(112–276)-7KA, in which all 7 lysine residues in this fragment (Lys at positions 121, 202, 225, 236, 240, 245, and 254) were substituted simultaneously with alanine (9). Some of these lysine residues (Lys^{240} and Lys^{245}) were implicated previously
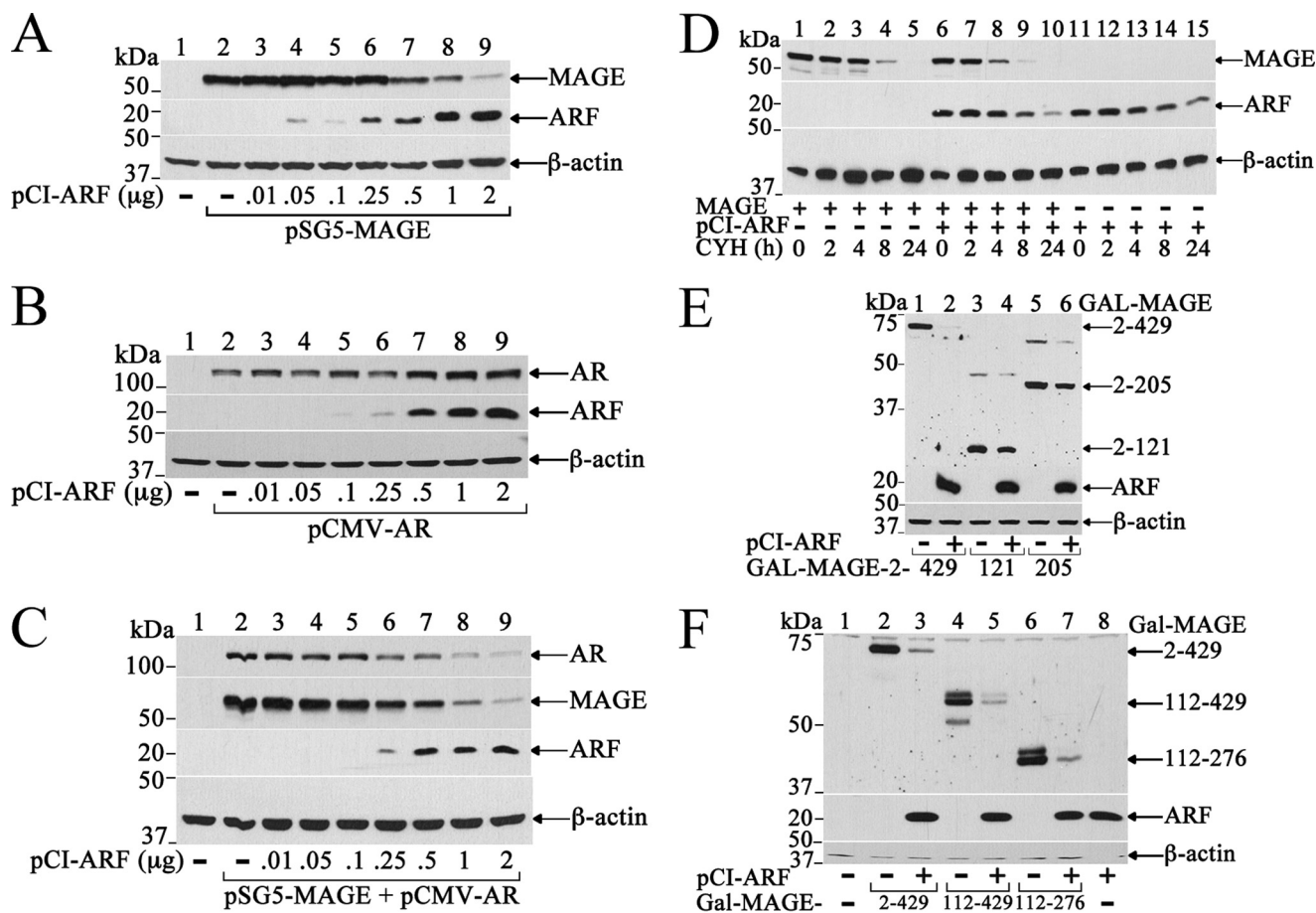
FIGURE 2. **ARF promotes MAGE-A11 degradation.** *A*, inverse relationship between MAGE-A11 and ARF was demonstrated by expressing 2 μg of pSG5 (*lane 1*) or 2 μg of pSG5-MAGE with 0.01–2 μg of pCI-neo-HA-ARF/two 6-cm CV1 cell dishes (*lanes 2–9*). Cell extracts (80 μg of protein/lane) pooled from two 6-cm dishes were analyzed on an immunoblot using MAGE1, HA, and β-actin antibodies. *B*, increase in AR and ARF was shown by expressing 2 μg of pCMV5 (*lane 1*) or 2 μg of pCMV-AR with 0.01–2 μg of pCI-neo-HA-ARF balanced with up to 2 μg of pCMV5 (*lane 2–9*)/two 6-cm CV1 cell dishes. Cell extracts (80 μg of protein/lane) from two pooled 6-cm dishes were probed on an immunoblot using AR32, HA, and β-actin antibodies. *C*, that ARF promotes AR degradation in the presence of MAGE-A11 was shown by expressing 2 μg of pSG5 (*lane 1*) or 2 μg of pSG5-MAGE with 1 μg of pCMV-AR and 0.01–2 μg of pCI-neo-HA-ARF/two 6-cm CV1 cell dishes (*lanes 2–9*). Cell extracts (80 μg of protein/lane) pooled from two 6-cm dishes were analyzed on an immunoblot using AR32, MAGE1, HA, and β-actin antibodies. *D*, increase in MAGE-A11 degradation in the presence of ARF was shown by expressing 1 μg of pSG5-MAGE with or without 0.5 μg of pCI-neo-HA-ARF/6-cm COS1 cell dish. The day after transfection, cells were incubated for 24 h in serum-free medium and then in fresh serum-free medium containing 10 μg/ml cycloheximide for 0, 2, 4, 8, and 24 h. Cell extracts (40 μg of protein/lane) were probed on an immunoblot using MAGE1, HA, and β-actin antibodies. *E*, region of MAGE-A11 required for degradation promoted by ARF was determined by expressing 5 μg of full-length GAL-MAGE-(2–429) or GAL-MAGE-(2–121) or 2–205 with or without 2 μg of pCI-neo-HA-ARF/6-cm COS1 cell dish. Cell extracts (60 μg of protein/lane) were probed on an immunoblot using GAL4 DNA-binding domain, HA, and β-actin antibodies. *F*, susceptibility of GAL-MAGE fragments to degradation promoted by ARF was tested by expressing 6 μg of GAL-O (*lane 1*), GAL-MAGE-(2–429) (*lanes 2* and *3*), 112–429 (*lanes 4* and *5*), or 112–276 (*lanes 6* and *7*) with or without 2 μg of pCI-neo-HA-ARF or pCI-neo-HA-ARF alone (*lane 8*) in 6-cm COS cell dishes. Cells were incubated with 1 μM MG132 for 20 h prior to harvest to increase detection of GAL-MAGE fragments. Cell extracts (80 μg of protein/lane) were probed on immunoblots using GAL DNA-binding domain, HA and β-actin antibodies.

as sites of monoubiquitination required for MAGE-A11 to interact with AR (9). The similar decline in MAGE-(112–276) and MAGE-(112–276)-7KA in the presence of ARF (Fig. 3*D*) provided evidence that lysine ubiquitination is not required for the proteasomal degradation of MAGE-A11 promoted by ARF. The difference in migration between MAGE-(112–276) and 7KA mutant was attributed to neutralization of multiple charged lysine residues by alanine substitution. Site-directed mutagenesis at hydrophobic residues Val[252], Ile[253], Leu[274], Phe[260], Phe[264], or Phe[275]; charged residue Arg[235]; or multiple lysines did not diminish MAGE-112–276 interaction with ARF.

The results suggest that the interaction between ARF and the central region of MAGE-A11 involves multiple domains that result in degradation by the proteasome independent of lysine ubiquitination. The ability of ARF to promote proteasomal degradation of MAGE-A11 is consistent with tumor suppressor of

ARF that inhibits the cell growth promoting properties of MAGE-A11.

Inverse Relationship between Endogenous ARF and MAGE-A11—An increase in MAGE-A11 degradation promoted by ARF should decrease endogenous MAGE-A11 levels in normal cells that have higher levels of ARF. Similarly, higher levels of endogenous MAGE-A11 in prostate cancer cells may be associated with low levels of ARF to promote the growth of castration-resistant prostate cancer. We therefore investigated the relative levels of endogenous MAGE-A11 and ARF.

ARF was not easy to detect in LAPC-4 prostate cancer cells that have relatively high levels of MAGE-A1 using human ARF and MAGE-A11-specific antibodies (Fig. 4*A*, *lane 4*) (2, 5). However, a weak band corresponding to ARF could be detected with overexposure of the blot (not shown), which indicated a low level of ARF in LAPC-4 cells. An inverse relationship
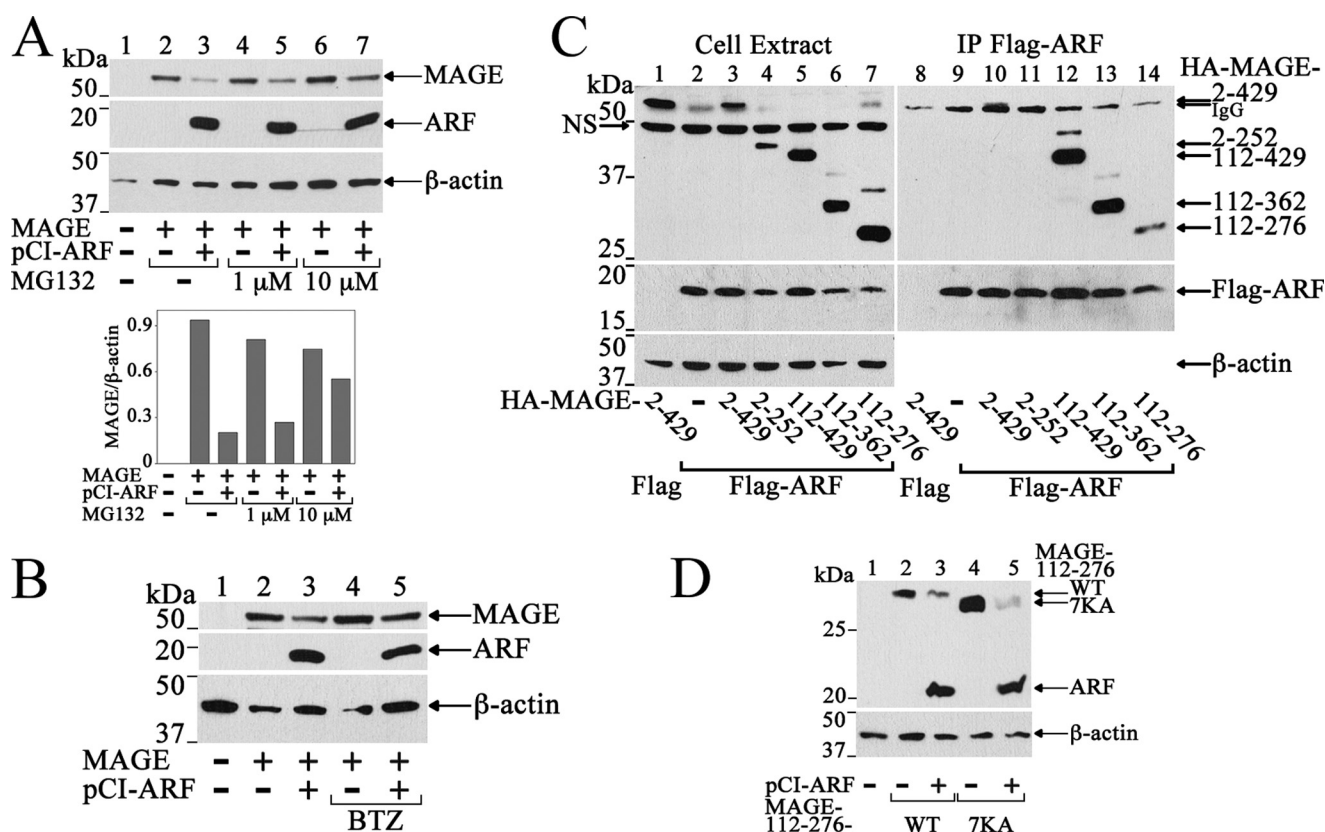
FIGURE 3. **Degradation of MAGE-A11 promoted by ARF mediated by the proteasome.** *A*, proteasomal degradation of MAGE-A11 promoted by ARF was shown by expressing 2 μg of pSG5 (*lane 1*) or 2 μg of pSG5-MAGE with or without 2 μg of pCI-neo-HA-ARF/6-cm COS1 cell dish treated for 2 h prior to harvest without (*lanes 2 and 3*) or with 1 μM MG132 (*lanes 4 and 5*) or 10 μM MG132 (*lanes 6 and 7*). The immunoblot of cell extracts (40 μg of protein/lane) was probed using MAGE1, HA, and β-actin antibodies. MAGE-A11 *versus* β-actin band intensity is shown in the *lower panel. B*, proteasomal degradation of MAGE-A11 promoted by ARF was tested by expressing 2 μg of pSG5 (*lane 1*) or 2 μg of pSG5-MAGE with or without 0.5 μg of pCI-neo-HA-ARF/6-cm COS1 cell dish treated for 4 h prior to harvest without (*lanes 2 and 3*) or with 5 μM bortezomib (LC Laboratories) (*lanes 4 and 5*). The immunoblot of cell extracts (80 μg of protein/lane) was probed using MAGE1, HA, and β-actin antibodies. *C*, coimmunoprecipitation of HA-MAGE fragments with FLAG-ARF was performed in COS1 cells by expressing 6 μg of FLAG empty vector or 6 μg of FLAG-ARF with 6 μg of pSG5-HA-MAGE-(2– 429), 3 μg of pSG5-HA-MAGE-(2–252), 3 μg of pSG5-HA-MAGE-(112– 429), 5 μg of pSG5-HA-MAGE-(112–362), or 6 μg of pSG5-HA-MAGE-(112–276). Cells were incubated for 24 h prior to harvest with 5 ng/ml EGF and 1 μM MG132. Cell extracts (60 μg of protein/lane) and immunoprecipitates were probed on the immunoblot using FLAG-M2, HA, and β-actin antibodies. *D*, independence of lysine ubiquitination for MAGE-A11 degradation promoted by ARF was demonstrated by expressing 6 μg of pSG5 (*lane 1*), pSG5-HA-MAGE-(112–276) WT (*lanes 2 and 3*), or pSG5-HA-MAGE-(112–276)-7KA with K121A,K202A,K225A,K236A,K240A,K245A,K254A mutations (*lanes 4 and 5*) with or without 2 μg of pCI-neo-HA-ARF/COS1 cell 6-cm dish. Cell extracts (40 μg of protein/lane) were probed on the immunoblot using HA and β-actin antibodies.
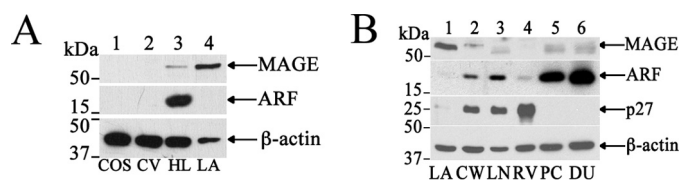
FIGURE 4. **Endogenous MAGE-A11 and ARF in cancer cells.** *A*, relative MAGE-A11 and ARF protein levels were compared in COS1, CV1, HeLa, and LAPC-4 cell extracts (80 μg of protein/lane) on immunoblots probed using MAGE1, ARF, and β-actin antibodies. *B*, relative MAGE-A11, ARF and p27[Kip1] protein levels in LAPC-4 (*LA*), CWR-R1 (*CW*), LNCaP (*LN*), 22Rv1 (*RV*), PC-3 (*PC*), and DU145 (*DU*) cell extracts (60 μg of protein/lane) probed using MAGE1, ARF, p27[Kip1], and β-actin antibodies.

between MAGE-A11 and ARF was also suggested by relatively low levels of MAGE-A11 and higher levels of ARF in human cervical carcinoma HeLa cells compared with LAPC-4 cells (Fig. 4*A*, *lanes 3 and 4*). ARF and MAGE-A11 were not detected in COS1 or CV1 cells (Fig. 4*A*, *lanes 1 and 2*), although endogenous MAGE-A11 can be detected in COS1 cells in a cell cycle-dependent manner (10).

PC-3 and DU145 prostate cancer cells had higher levels of ARF (Fig. 4*B*, *lanes 5 and 6*) (44, 45), and MAGE-A11 was not

detected, consistent with ARF promoting the degradation of MAGE-A11. Low levels of MAGE-A11 in PC-3 and DU145 cells also result from CpG dinucleotide DNA methylation at the *MAGE-A11* gene promoter transcription start site (2). LNCaP, CWR-R1, and 22Rv1 prostate cancer cells had intermediate levels of ARF relative to LAPC-4, PC-3, and DU145 cells, and MAGE-A11 was difficult to detect (Fig. 4*B*).

The cyclin-dependent kinase inhibitor and tumor suppressor p27[Kip1] is down-regulated by MAGE-A11 in LAPC-4 cells (5). Accordingly, p27[Kip1] was almost undetectable in LAPC-4 and was not detected in PC-3 or DU145 cells (Fig. 4*B*). A similar intermediate level of p27[Kip1] in CWR-R1 and LNCaP cells was less than 22Rv1 cells.

The results demonstrate that higher levels of MAGE-A11 are associated with low levels of ARF in support of ARF promoting the degradation of MAGE-A11. The results provide further evidence that MAGE-A11 contributes to low levels of p27[Kip1] and that p27[Kip1] may be down-regulated by ARF independent of MAGE-A11.

Down-regulation of MAGE-A11 by ARF—To address further a possible inverse relationship between MAGE-A11 and ARF,
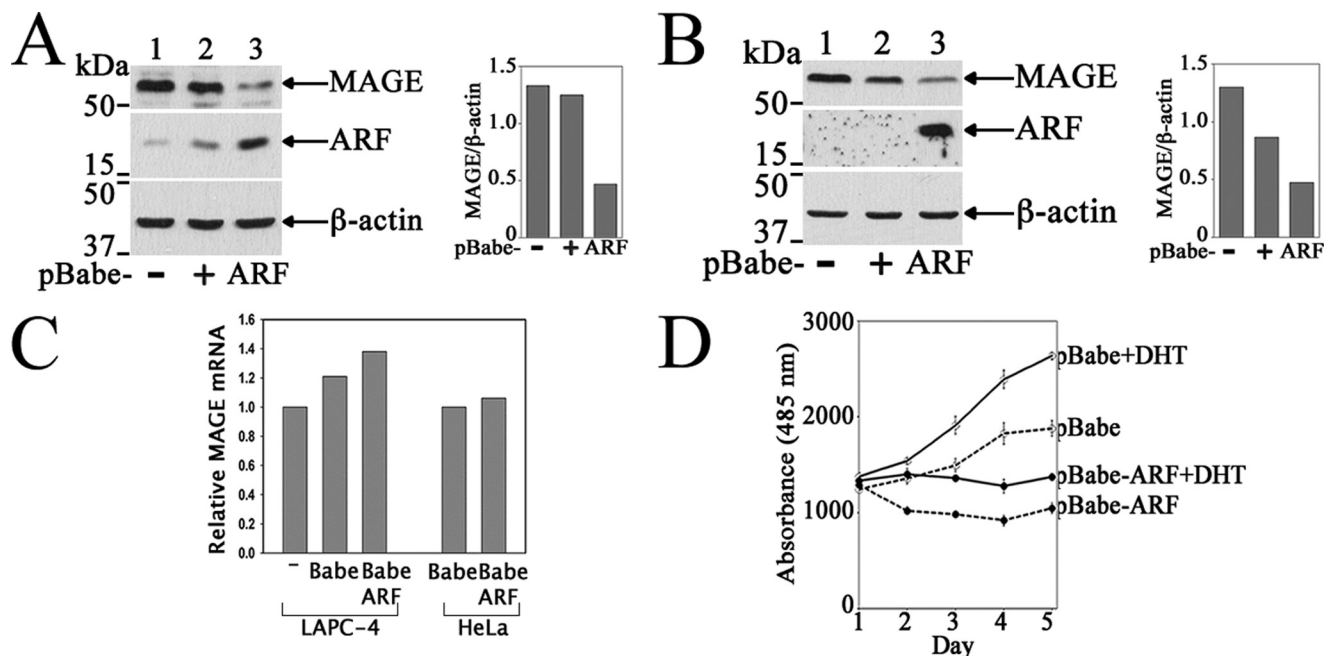
FIGURE 5. **Down-regulation of endogenous MAGE-A11 by ARF.** *A*, down-regulation of MAGE-A11 in HeLa cells by ARF was shown using untransduced HeLa cells (*lane 1*), HeLa cells transduced with 0.25 ml of pBabe-puro control retrovirus/well of a 6-well plate (*lane 2*), or pBabe-puro-hARF retrovirus for stable expression of human ARF (*lane 3*). Retrovirus transduced cells were selected using 3 μg/ml puromycin as described under "Experimental Procedures." Cell extracts (60 μg of protein/lane) were probed on the immunoblot using MAGE1 (10 μg/ml), human p14-ARF 10437 (1:500 dilution), and β-actin antibodies. MAGE-A11 *versus* β-actin band intensity is shown in the *right panel*. *B*, down-regulation of MAGE-A11 in LAPC-4 cells by ARF was shown using untransduced LAPC-4 cells (*lane 1*), LAPC-4 cells transduced with 0.25 ml pBabe-puro-derived control retrovirus/well of a 6-well plate (*lane 2*), or pBabe-puro-hARF retrovirus for stable expression of human ARF (*lane 3*). Retrovirus transduced LAPC-4 cells were selected using 3 μg/ml puromycin as described under "Experimental Procedures." Cell extracts (60 μg of protein/lane) were probed on the immunoblot using MAGE1 (10 μg/ml), human p14-ARF 10437 (1:500 dilution), and β-actin antibodies. MAGE-A11 *versus* β-actin band intensity is shown in the *right panel*. *C*, quantitative RT-PCR was performed on RNA extracted from LAPC-4 or HeLa pBabe-control or pBabe-ARF expressing cells using specific amplification primers and shown for MAGE-A11 relative to the peptidylprolyl isomerase A control. *D*, increased ARF expression slows LAPC-4 cell growth was shown using LAPC-4 cells transduced with retrovirus as described in *B*. The day after plating, cells were incubated with or without 1 nM DHT and quantitated daily using a colorimetric assay as described under "Experimental Procedures." Statistical significance between LAPC-4 pBabe and pBabe-ARF cells was observed in the absence or presence of 1 nM DHT based on two-way ANOVA ($p < 0.001$).

retrovirus derived from pBabe-control and pBabe-ARF vectors were used for stable expression of ARF in HeLa and LAPC-4 cells selected using puromycin. A ~3-fold or greater increase in ARF decreased endogenous MAGE-A11 levels by 64% in HeLa cells (Fig. 5*A*) and by 45% in LAPC-4 cells (Fig. 5*B*) relative to control cells and normalized to the β-actin protein loading control. Quantitative RT-PCR analysis of MAGE-A11 mRNA extracted from LAPC-4 and HeLa pBabe-control or pBabe-ARF expressing cells using MAGE-A11 and peptidylprolyl isomerase A control specific primers (38) showed no significant change in MAGE-A11 mRNA with the expression of ARF (Fig. 5*C*). The results demonstrate that lower levels of MAGE-A11 are associated with higher levels of ARF in support of an inverse relationship between MAGE-A11 and ARF consistent with MAGE-A11 degradation promoted by ARF.

We next determined whether stable retrovirus expression of ARF alters the growth of LAPC-4 cells in the absence or presence of androgen. DHT increased the growth of LAPC-4 pBabe-control cells analyzed using a colorimetric cell counting assay (Fig. 5*D*). However, stable expression of ARF inhibited LAPC-4 cell growth over 5 days in the absence or presence of 1 nM DHT.

An increase in MAGE-A11 degradation promoted by ARF would suggest that knockdown of ARF might increase endogenous levels of MAGE-A11. This was explored in HeLa cells that express low levels of MAGE-A11 and higher levels of ARF rel-

ative to LAPC-4 cells. Lentivirus-expressed shRNA that targets the unique exon 1B region of the ARF gene decreased ARF levels and increased the levels of MAGE-A11 in HeLa cells treated without (Fig. 6*A*) or with the proteasome inhibitor, MG132 (Fig. 6*B*).

The results suggest that the low abundance MAGE-A11 regulatory protein is inversely regulated to ARF. Increased degradation of MAGE-A11 promoted by ARF was associated with inhibition of prostate cancer cell growth.

Inhibition of AR Transcriptional Activity by Human ARF— The inhibitory effect of ARF on coregulator activity of MAGE-A11 was investigated by measuring AR transcriptional activity that is increased by MAGE-A11 (1). AR transactivation of the prostate-specific antigen enhancer linked to a luciferase reporter gene was inhibited by ARF (Fig. 7*A*) in agreement with a previous report (46). ARF also blocked the stimulatory effect of MAGE-A11 on androgen-dependent AR transcriptional activity. The results suggest that an inhibitory effect of ARF on AR transcriptional activity is mediated by negative regulation of MAGE-A11.

Inhibition by ARF of the MAGE-A11-induced increase in AR transactivation suggested that the interaction between AR and MAGE-A11 does not protect against degradation promoted by ARF. This was suggested earlier (Fig. 2) where susceptibility of MAGE-A11 to degradation promoted by ARF was extended to AR. ARF also inhibited the stabilization of MAGE-A11 in the
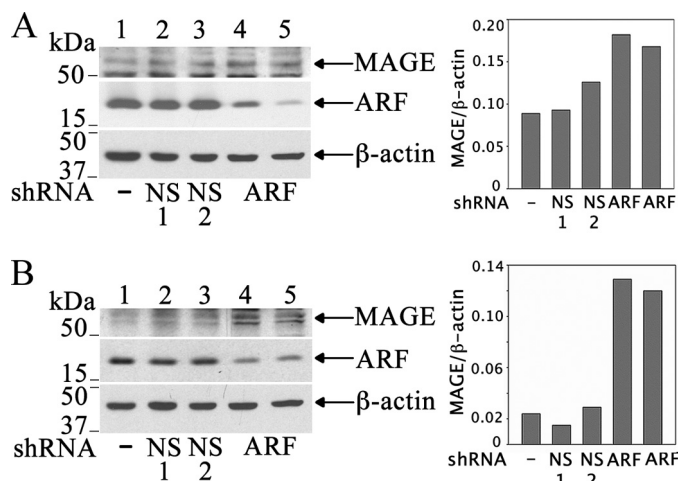
FIGURE 6. **Lentivirus shRNA knockdown of ARF increases MAGE-A11 levels.** HeLa cells in 6-well plates were untransduced (−) or transduced with nonspecific control lentivirus shRNA for empty shRNA (*NS1*), 18-bp scrambled lentivirus nonspecific control-2 (*NS2*), or 100 or 250 μl of lentivirus ARF shRNA that targets exon 1B of the ARF gene. Cells were selected using 3 μg/ml puromycin and incubated (*A*) without or (*B*) with 1 μM MG132 proteasome inhibitor 20 h prior to harvest. Immunoblots of cell extracts (60 μg of protein/lane) were probed using FLAG-MAGE1, ARF, and β-actin antibodies. MAGE-A11 to β-actin relative band intensity is shown on the *right*.

presence of AR (Fig. 7*B*, *lanes 3–6*) and interfered with the increase in AR levels in the presence of MAGE-A11 (Fig. 7*B*, *lanes 2–4*). Reciprocal stabilization between AR and MAGE-A11 in the absence or presence of androgen only partially rescued MAGE-A11 from degradation promoted by ARF (Fig. 7*C*).

A similar analysis was performed on the constitutive activity of AR-(1–660) NH$_2$-terminal and DNA-binding fragment that lacks the ligand binding domain and mimics AR splice variants reported in prostate cancer (47–49). ARF inhibited the constitutive activity of AR-(1–660) with or without expression of MAGE-A11 (Fig. 8*A*, *left bars*). However, constitutive activity of AR-(1–660)-L26A,F27A, in which the AR NH$_2$-terminal F*XX*LF motif interaction site for MAGE-A11 was mutated (1), showed little stimulation by MAGE-A11 or inhibition by ARF (Fig. 8*A*, *right bars*).

The increase in AR-(1–660) constitutive activity in the presence of MAGE-A11 was associated with reciprocal stabilization between AR-(1–660) and MAGE-A11 (Fig. 8, *B* and *C*) similar to that seen with full-length AR. The increase in AR-(1–660) levels associated with MAGE-A11 was blocked by ARF, and MAGE-A11 interaction with AR-(1–660) only partially rescued MAGE-A11 from degradation promoted by ARF. The absence of a stabilizing effect of MAGE-A11 on AR-(1–660)-L26A,F27A (Fig. 8*C*) demonstrated reciprocal stabilization between AR and MAGE-A11 depended on MAGE-A11 binding the AR NH$_2$-terminal F*XX*LF motif.

The results suggest that ARF interferes with MAGE-A11-induced AR stabilization and increased transcriptional activity of AR or a splice variant-like form of AR. MAGE-A11 remained susceptible to degradation promoted by ARF independent of its interaction with AR.

Human ARF Inhibits the MAGE-A11-induced Increase in E2F1 Transcriptional Activity—MAGE-A11 interacts with, stabilizes, and increases the transcriptional activity of E2F1, a transcription factor that promotes progression through the cell

cycle (5). In contrast, ARF interacts with and inhibits E2F1 transcriptional activity through unknown mechanisms (42). We therefore determined whether ARF blocks the stabilization of E2F1 by MAGE-A11 or the MAGE-A11-dependent increase in endogenous E2F1 transcriptional activity.

The increase in E2F1 levels associated with MAGE-A11 was partially inhibited by ARF (Fig. 9*A*, *lanes 4–6*). This did not appear to result simply from increased degradation of MAGE-A11 promoted by ARF. MAGE-A11 levels declined to a greater extent with ARF expression than with E2F1 stabilization (Fig. 9*A*, *lanes 2–5*). However, ARF did not decrease MAGE-A11 levels further in the presence of E2F1, and ARF did not promote the degradation of E2F1, in agreement and in contrast to previous reports (41, 51).

Immunoprecipitation studies addressed whether the inhibitory effects of ARF on E2F1 stabilization by MAGE-A11 might result from ARF interference with MAGE-A11 interaction with E2F1. MAGE-A11 and ARF were each associated with FLAG-E2F1 (Fig. 9*B*, *lanes 5* and *6*). However, when expressed together, ARF inhibited the interaction between MAGE-A11 and E2F1 to a greater extent than could be accounted for by the lower levels of MAGE-A11 in the presence of ARF (Fig. 9*B*, *lane 7*). ARF also blocked the dose-dependent stimulatory effect of MAGE-A11 on endogenous E2F1 transcriptional activity when assayed in the absence or presence of p300 (Fig. 9*C*). There was a dose-dependent decrease in MAGE-A11-induced endogenous E2F1 transcriptional activity with increasing expression of ARF (Fig. 9*D*). Specificity for transcriptional repression by ARF was suggested by the absence of inhibition by ARF of CMV-Luc or pSG5-Luc constitutive transcriptional activity (not shown).

The results suggest that ARF interferes with the stabilizing and transcriptional enhancing effects of MAGE-A11 on E2F1 by blocking MAGE-A11 interaction with E2F1 and by promoting MAGE-A11 degradation. Inhibition of the stimulatory effects of MAGE-A11 on E2F1 transcriptional activity is consistent with the tumor suppressor activity of ARF.

Effects of MAGE-A11 on HDM2 Stabilization and E2F1 Transcriptional Activity—A well known function of ARF that contributes to cell cycle arrest is the inhibition of HDM2 E3 ubiquitin ligase activity, which results in up-regulation of p53 to promote cell cycle arrest or apoptosis (23–27, 52) and down-regulation of E2F1 activation of genes involved in cell cycle progression (53–55). This suggests that one mechanism by which cancer cells interfere with the tumor suppressor activity of ARF is through the stabilization of HDM2.

We found that MAGE-A11 increased the levels of ~90-kDa HDM2 by ~15-fold detected on an 8% acrylamide gel and an up-shifted self-ubiquitinated or sumoylated form of HDM2 (56, 57) when normalized to the β-actin protein loading control (Fig. 10*A*). Stabilization of ~100-kDa HDM2 by MAGE-A11 on a 12% acrylamide gel was also increased in the presence of ARF (Fig. 10*B*, *lanes 2–5*), even though ARF promoted the degradation of MAGE-A11 in the absence or presence of HDM2 (Fig. 10*B*, *lanes 5–7*). The small increase in HDM2 by ARF (Fig. 10*B*, *lanes 2* and *4*) was in agreement with a previous report that ARF increases the steady-state levels of HDM2 (58). Although stabilization of HDM2 by MAGE-A11 was inhibited by E2F1 (Fig. 10*C*, *lanes 5* and *7*), there was no evidence that HDM2 altered
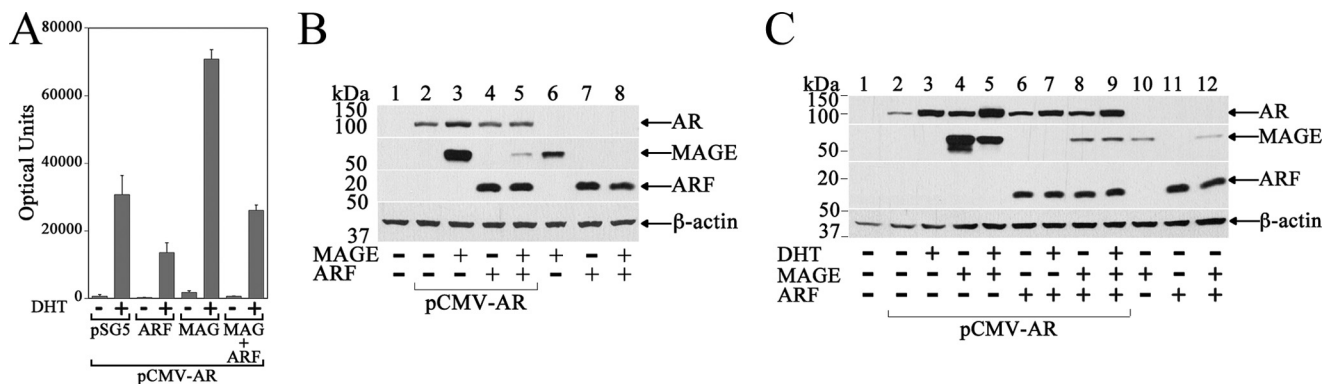
FIGURE 7. ARF inhibition of androgen-dependent AR transcriptional activity and reciprocal AR and MAGE-A11 stabilization did not protect MAGE-A11 from degradation promoted by ARF. *A*, AR transcriptional activity was assayed in 12-well CV1 cell plates using 0.25 μg of PSA-Enh-Luc and 25 ng of pCMV-AR with 50 ng of pSG5 or 25 ng of pCI-neo-HA-ARF and 50 ng of pSG5-MAGE alone or together. The cells were incubated for 24 h with or without 10 nM DHT. *B*, immunoblot showing AR does not protect MAGE-A11 against degradation promoted by ARF was performed in 10-cm CV1 cell dishes by expressing 2 μg of pCMV5 (*lane 1*) or 2 μg of pCMV-AR (*lane 2*) with 1 μg of pSG5-MAGE (*lane 3*), 2 μg of pCI-neo-HA-ARF (*lane 4*), or pSG5-MAGE and pCI-neo-HA-ARF together (*lane 5*). pSG5-MAGE (1 μg, *lane 6*) or 2 μg of pCI-neo-HA-ARF was expressed alone (*lane 7*) or together (*lane 8*). The immunoblot of cell extracts (80 μg of protein/lane) was probed using AR32, MAGE1, HA, and β-actin antibodies. *C*, androgen dependence of AR and ARF effects on MAGE-A11 was performed using pCMV5 (*lane 1*) or pCMV-AR alone with or without 10 nM DHT (*lanes 2 and 3*) or pCMV-AR with pSG5-MAGE with or without 10 nM DHT (*lanes 4 and 5*), pCI-neo-HA-ARF with or without 10 nM DHT (*lanes 6 and 7*), pSG5-MAGE and pCI-neo-HA-ARF with or without 10 nM DHT (*lanes 8 and 9*), pSG5-MAGE (*lane 10*), pCI-neo-HA-ARF (*lane 11*), or pSG5-MAGE and pCI-neo-HA-ARF (*lane 12*) in CV1 cells as described and probed in *B*.
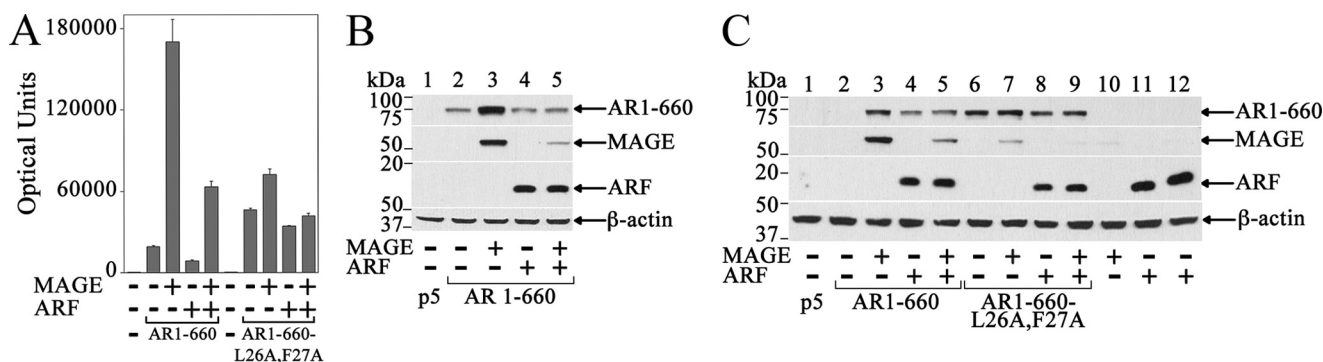
FIGURE 8. Inhibition of AR-(1–660) constitutive activity by ARF. *A*, effect of ARF on constitutive activity of AR-(1–660) and AR-(1–660)-F*XX*AA was determined in 12-well CV1 cell plates by expressing 0.25 μg of PSA-Enh-Luc with 25 ng of pCMV5 (−), pCMV-AR-(1–660) or pCMV-AR-(1–660)-L26A,F27A with 50 ng of pSG5 (−), 50 ng of pSG5-MAGE, or 25 ng of pCI-neo-HA-ARF alone or together. *B*, ARF inhibition of AR-(1–660) stabilization by MAGE-A11 was shown on an immunoblot by expressing 2 μg of pCMV5 (*lane 1*) or 2 μg of pCMV-AR-(1–660) alone (*lane 2*) or with 1 μg of pSG5-MAGE (*lane 3*), 2 μg of pCI-neo-HA-ARF (*lane 4*), or pSG5-MAGE and pCI-neo-HA-ARF together (*lane 5*) per 10-cm CV1 cell dish. The immunoblot of cell extracts (80 μg of protein/lane) was probed using AR32, MAGE1, HA, and β-actin antibodies. *C*, ARF inhibition of AR-(1–660) stabilization by MAGE-A11 is shown on an immunoblot of AR-(1–660) or AR-(1–660)-L26A,F27A mutant, MAGE-A11, and ARF performed in CV1 cells and probed as in *B*.

E2F1 levels, and HDM2 did not interfere with E2F1 stabilization by MAGE-A11 (Fig. 10*C*, *lanes 2–5*).

The increase in HDM2 caused by MAGE-A11 (Fig. 10*A*) and the ability of MAGE-A11 or HDM2 to increase E2F1 transcriptional activity (5, 53–55) suggested that MAGE-A11 and HDM2 may cooperate to up-regulate E2F1 transcriptional activity. This was supported by a similar increase in endogenous E2F1 transactivation in response to HDM2 or MAGE-A11 that was nearly additive when expressed together (Fig. 10*D*). MAGE-A11 and HDM2 functioned cooperatively with p300 to up-regulate endogenous E2F1 transcriptional activity, which reflects the ability of MAGE-A11 and HDM2 to interact with p300 during gene transcription (5, 10, 59). The increase in E2F1 transcriptional activity in response to MAGE-A11 and p300 was greater with HDM2 and inhibited by ARF with or without HDM2 (Fig. 10*E*). Cooperation between MAGE-A11, HDM2, and p300 in the up-regulation of E2F1 transcriptional activity suggested that p300 might influence HDM2 levels. However, unlike MAGE-A11, which stabilized HDM2 (Fig. 10*F*, *lanes 2 and 3*), p300 did not increase HDM2 or interfere with HDM2

stabilization in the presence of MAGE-A11 (Fig. 10*F*, *lanes 3–5*).

The results suggest that MAGE-A11 cooperates with HDM2 and p300 to up-regulate E2F1 transcriptional activity. MAGE-A11 in a complex with HDM2 and ARF may interfere with HDM2 inactivation by ARF.

Discussion

Post-translational Down-regulation of MAGE-A11 by Human ARF Tumor Suppressor—MAGE-A11 is a multifunctional protein involved in the regulation of cell cycle proteins. The evolution of the *MAGE-A11* among primates, its increased expression during androgen deprivation therapy of prostate cancer, its function as an AR coregulator, and the requirement for MAGE-A11 in prostate cancer cell growth support the concept that *MAGE-A11* is a proto-oncogene that hyperactivates human AR and promotes the development of castration-resistant prostate cancer (38). One mechanism for the increase in MAGE-A11 in prostate cancer clinical samples during androgen deprivation therapy and in the CWR22 human xenograft
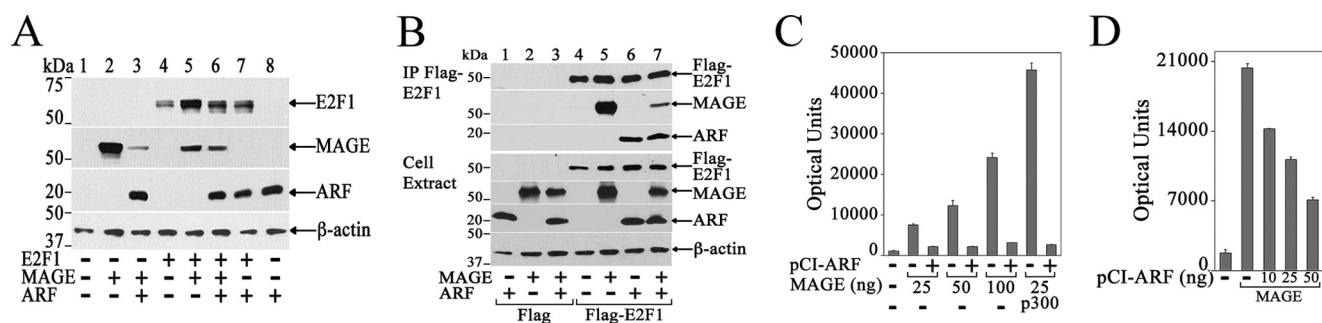
FIGURE 9. ARF inhibits MAGE-A11 interaction with E2F1. *A*, ARF inhibition of E2F1 stabilization by MAGE-A11 was shown by expressing 2 μg of pSG5 (*lane 1*) or 2 μg of pSG5-MAGE without (*lane 2*) or with 2 μg of pCI-neo-HA-ARF (*lane 3*) per 10-cm CV1 cell dish. pCMV-E2F1 (1 μg) was expressed alone (*lane 4*) or with 2 μg of pSG5-MAGE (*lane 5*), 2 μg of pSG5-MAGE, and 2 μg of pCI-neo-HA-ARF (*lane 6*) or 2 μg of pCI-neo-HA-ARF (*lane 7*). pCI-neo-HA-ARF (2 μg) was expressed alone (*lane 8*). The immunoblot of cell extracts (80 μg of protein/lane) was probed using E2F1, MAGE1, HA, and β-actin antibodies. *B*, ARF inhibition of MAGE-A11 interaction with E2F1 was shown by coimmunoprecipitation of MAGE-A11 and ARF with FLAG-E2F1 using 4 μg of pCMV-FLAG (*lanes 1–3*) or pCMV-FLAG-E2F1 (*lanes 4–7*) alone (*lane 4*) or with 6 μg of pCI-neo-HA-ARF (*lanes 1 and 6*), 4 μg of pSG5-MAGE (*lanes 2 and 5*), or pCI-neo-HA-ARF and pSG5-MAGE (*lanes 3 and 7*) per 10-cm COS1 cell dish. Immunoprecipitates (*IP*, *top three panels*) and cell extracts (60 μg of protein/lane, *bottom four panels*) were probed using MAGE2 antibody that does not cross-react with FLAG, FLAG-M2 for FLAG-E2F1, HA antibody for HA-ARF, and β-actin antibodies. *C*, inhibition by ARF of endogenous E2F1 transcriptional activity stimulated by MAGE-A11 was measured in HeLa cells using 0.1 μg of E2F1-Luc in the presence of 0.1 μg of pSG5 empty vector (−) or 25, 50, or 100 ng of pSG5 (−) or pSG5-MAGE with or without 50 ng of pCI-neo-HA-ARF and/or 25 ng of pSG5-HA-p300. *D*, dose-dependent ARF inhibition of MAGE-A11 stimulation of endogenous E2F1 transcriptional activity was determined in HeLa cells using 0.1 μg of E2F1-Luc and 0.1 μg of pSG5 (−) or 0.1 μg of pSG5-MAGE expressed with 50 ng of pCMV5 (−) or 10, 25, or 50 ng of pCI-neo-HA-ARF.

model of prostate cancer that undergoes remission after castration but regrows after castration is progressive hypomethylation of CpG dinucleotides at the transcription start site of the *MAGE-A11* gene promoter (2, 3). *MAGE-A11* expression is also up-regulated in prostate cancer during androgen deprivation therapy by increasing levels of cAMP associated with down-regulation of phosphodiesterases that degrade cAMP (2, 60–63). In this report, we extend the family of MAGE-A11 interacting partners to include the human ARF tumor suppressor that targets MAGE-A11 for degradation by the proteasome independent of lysine ubiquitination.

Our studies suggest that down-regulation of MAGE-A11 by ARF represents a third mechanism that controls MAGE-A11, where low levels of ARF contribute to higher levels of MAGE-A11 during prostate cancer progression. Our findings are consistent with the tumor suppressor activity of ARF that protects normal cells from tumorigenesis and the proto-oncogene activity of MAGE-A11 that increases prostate cancer cell growth. The increase in MAGE-A11 in prostate cancer during androgen deprivation therapy provides an escape mechanism whereby prostate cancer cells survive and expand in an environment of low intratumoral active androgen biosynthesis.

Our studies suggest a model (Fig. 11) in which MAGE-A11 is central to a protein network involved in human cell growth regulation. We showed previously that MAGE-A11 increases AR transcriptional activity by binding the AR NH$_2$-terminal F*XX*LF motif and recruiting p300 and TIF2 (NCOA2) transcriptional coregulators, which increases androgen-dependent prostate cancer cell growth (1, 10, 11, 38). Here we provide evidence that MAGE-A11 interacts with and is targeted for degradation by the proteasome by the human ARF tumor suppressor. MAGE-A11 functions cooperatively with HDM2 E3 ubiquitin ligase to increase the transcriptional activity of E2F1, an oncoprotein that promotes progression through the cell cycle (5, 64). ARF inhibits the increase in AR and E2F1 transcriptional activity induced by MAGE-A11. Multiple interaction regions between ARF and HDM2 (65) were seen between MAGE-A11 and ARF that resulted in MAGE-A11 degradation.

Inhibition of the stimulatory effects of MAGE-A11 on AR and E2F1 transcriptional activity by ARF provides new mechanisms of tumor suppression and maintenance of the nontransformed phenotype (5, 42).

Low levels of ARF associated with cancer development result from destabilization of the ARF protein or from *INK4a/ARF* gene deletions, mutations, or methylation (66, 67). Low levels of ARF in prostate cancer (19–21) were also attributed to androgen-inducible microRNA-125b that negatively regulates ARF in prostate cancer (68). Our studies suggest that low levels of ARF in prostate cancer increase the levels of MAGE-A11 by decreasing MAGE-A11 degradation. Our findings are consistent with the proto-oncogene properties associated with increased levels of MAGE-A11 and with the tumor suppressor activity of ARF. Increased expression of ARF was proposed as a therapeutic approach to increase cancer sensitivity to chemotherapy (69). Our findings suggest that prostate cancers with elevated levels of MAGE-A11 are candidates for clinical intervention that increases ARF, which would decrease MAGE-A11 and induce cell cycle arrest or apoptosis. The association between higher levels of MAGE-A11 and low ARF in prostate cancer supports the concept that ARF maintains low levels of MAGE-A11 in normal cells by promoting degradation by the proteasome, and human ARF tumor suppressor activity depends on the post-translational down-regulation of MAGE-A11.

ARF Counteracts the Stimulatory Effects of MAGE-A11 on AR Transcriptional Activity—Increased AR transcriptional activity in response to low intratumoral androgen synthesis is a principal driver of castration-resistant prostate cancer (70, 71). AR transcriptional activity also increases in response to higher levels of MAGE-A11, which has been observed in ∼36% of castration-resistant prostate cancers (1, 2). Here we show that ARF interferes with the stimulatory effects of MAGE-A11 on androgen-dependent AR transcriptional activity and the constitutive activity of a splice-variant-like AR reported in prostate cancer (47–49). The findings suggest that the inhibitory effects
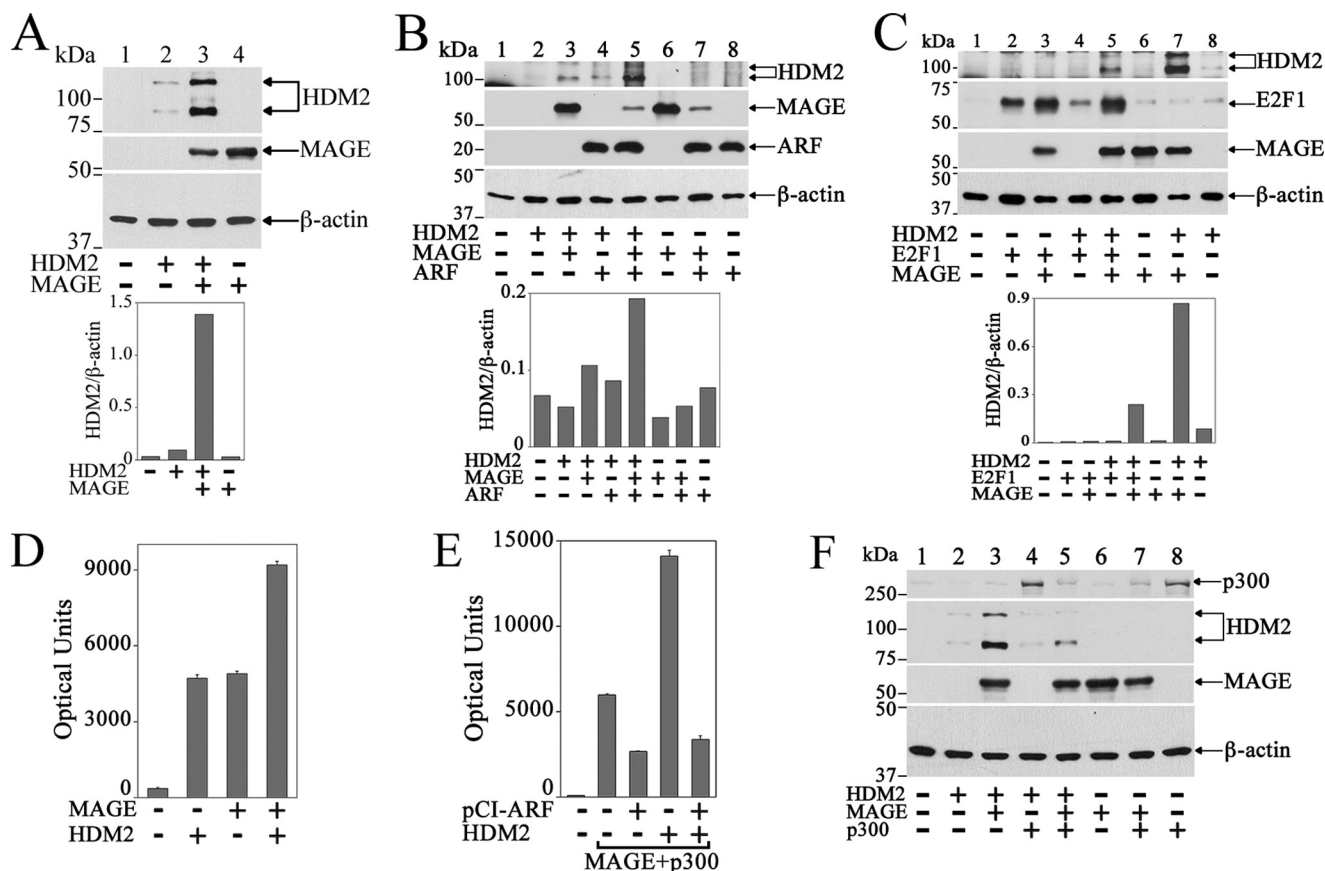
FIGURE 10. **Stabilization of HDM2 by MAGE-A11.** *A*, stabilization of HDM2 by MAGE-A11 was shown on an immunoblot by expressing 6 μg of pCMV5 (*lane 1*), 6 μg of pCMV-HA2-HDM2 alone (*lane 2*), or with 3 μg of pSG5-MAGE (*lane 3*) or 3 μg of pSG5-MAGE alone (*lane 4*) per 10-cm COS1 cell dish. Cells were treated with 5 ng/ml EGF for 24 h prior to harvest. The immunoblot of cell extracts (60 μg of protein/lane) from an 8% acrylamide gel was probed using HA, MAGE1, and β-actin antibodies. The higher molecular weight form of HDM2 results from self-ubiquitination or sumoylation (56, 57). The $\sim$90-kDa HDM2 *versus* β-actin band intensity is shown in the lower panel. *B*, HDM2 stabilization by MAGE-A11 in the presence of ARF was shown on an immunoblot using 6 μg of pCMV5 (*lane 1*), 6 μg of pCMV-HA2-HDM2 alone (*lane 2*) or with 3 μg of pSG5-MAGE (*lane 3*), or 3 μg of pCI-neo-HA-ARF alone (*lane 4*) or with pSG5-MAGE and pCI-neo-HA-ARF together (*lane 5*) per 10-cm COS1 cells. pSG5-MAGE (3 μg) was expressed alone (*lane 6*) or with 3 μg of pCI-neo-HA-ARF (*lane 7*), or pCI-neo-HA-ARF was expressed alone (*lane 8*). The immunoblot of cell extracts (50 μg of protein/lane) from a 12% acrylamide gel was probed using HA, MAGE1, and β-actin antibodies. The $\sim$100-kDa HDM2 *versus* β-actin band intensity is shown in the *lower panel*. *C*, E2F1 inhibition of HDM2 stabilization by MAGE-A11 was shown on an immunoblot by expressing 3 μg of pCMV5 (*lane 1*) or 3 μg of pCMV-E2F1 alone (*lane 2*) or with 3 μg of pSG5-MAGE (*lane 3*), 6 μg of pCMV-HA2-HDM2 (*lane 4*), or with pSG5-MAGE and pCMV-HA2-HDM2 together (*lane 5*). pSG5-MAGE (3 μg) was expressed alone (*lane 6*) or with 6 μg of pCMV-HA2-HDM2 (*lane 7*) or 6 μg of pCMV-HA2-HDM2 was expressed alone (*lane 8*) per 10-cm COS1 cell dish. The immunoblot of cell extracts (50 μg of protein/lane) from a 12% acrylamide gel was probed using HA, E2F1, MAGE1, and β-actin antibodies. The $\sim$100-kDa HDM2 *versus* β-actin band intensity is shown in the *lower panel*. *D*, additive effects of MAGE-A11 and HDM2 on endogenous E2F1 transcriptional activity was determined in HeLa cells by expressing 0.1 μg of E2F1-Luc with 25 ng of pSG5 with or without ($-$) 25 ng of CMV-HDM2 and 25 ng of MAGE-A11 alone or together. *E*, ARF inhibition of endogenous E2F1 transcriptional activity induced by MAGE-A11, HDM2, and p300 was determined in HeLa cells by expressing 0.1 μg of E2F1-Luc with 25 ng of pSG5 ($-$), or 25 ng of pSG5-MAGE and 50 ng of pSG5-HA-p300 with 50 ng of pCMV5 ($-$) or 50 ng of pCI-neo-HA-ARF, or 25 ng of pSG5-MAGE, 25 ng of pSG5-HA-p300, and 25 ng of pCMV-HDM2 with 50 ng of pCMV5 ($-$) or 50 ng of pCI-neo-HA-ARF. *F*, p300 inhibition of HDM2 stabilization by MAGE-A11 was shown by expressing 6 μg of pCMV5 (*lane 1*) or 6 μg of pCMV-HA2-HDM2 alone (*lane 2*) or with 3 μg of pSG5-MAGE (*lane 3*), 6 μg of pSG5-HA-p300 (*lane 4*) or pSG5-MAGE and pSG5-HA-p300 together (*lane 5*) per 10-cm COS1 cell dish. pSG5-MAGE was expressed alone (*lane 6*) or with pSG5-HA-p300 (*lane 7*), and pSG5-HA-p300 was expressed alone (*lane 8*). The immunoblot of cell extracts (60 μg of protein/lane) from an 8% acrylamide gel was probed using p300, HA, MAGE1, and β-actin antibodies.

of ARF on AR transcriptional activity are mediated in part by down-regulation of MAGE-A11.

It was reported that ARF inhibits AR transcriptional activity by interfering with the androgen-dependent AR NH$_2$- and COOH-terminal interaction (46) that stabilizes AR in the presence of AR agonists (14, 72) and that AR is degraded by the proteasome in association with gene activation (73). We found that ARF interferes with a positive relationship between AR and MAGE-A11 by targeting MAGE-A11 for degradation. Although ARF did not directly decrease AR levels, ARF negatively regulated AR in the presence of MAGE-A11. Dependence on MAGE-A11 interaction with the AR NH$_2$-terminal F*XX*LF motif for the effects of ARF provided evidence that ARF inter-

feres with AR transcriptional activity by negatively regulating MAGE-A11. The inhibitory effects of ARF on AR transcriptional activity evident without the increased expression of MAGE-A11 suggest that negative regulation of AR by ARF occurs even when MAGE-A11 levels are low (10).

Up-regulation of HDM2 by MAGE-A11 Increases E2F1 Transcriptional Activity That Is Inhibited by ARF—HDM2 E3 ubiquitin ligase is a proto-oncogene overexpressed in human cancers and a target for anticancer therapy (74, 75). HDM2 stabilizes E2F1 and mediates ubiquitin-dependent degradation of p53 (54). Here we show that MAGE-A11 cooperates with HDM2 to stabilize and increase the transcriptional activity of E2F1, an oncoprotein that up-regulates genes important for cell
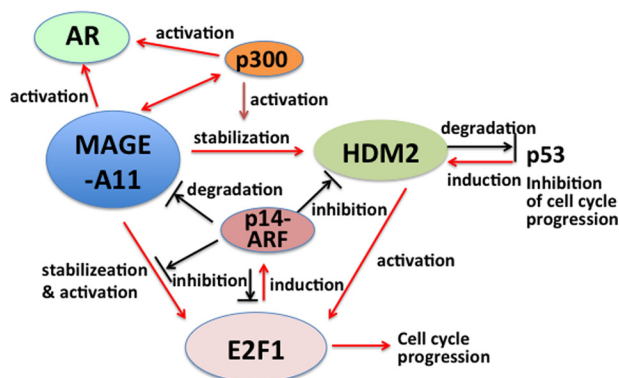
FIGURE 11. **Model of MAGE-A11 interaction with cell cycle regulators.** MAGE-A11 increases AR transcriptional activity by binding the AR NH_2-terminal FXXLF motif and recruiting p300, which promotes androgen-dependent prostate cancer cell growth (1, 10, 11, 63). MAGE-A11 stabilizes and increases the transcriptional activity of the E2F1 transcription factor and oncoprotein that promotes S phase entry into the cell cycle (5, 78). Human p14-ARF is a tumor suppressor that interacts with MAGE-A11 and promotes the degradation of MAGE-A11 by the proteasome independent of lysine ubiquitination. Inhibition by ARF of the stimulatory effects of MAGE-A11 on E2F1 may account for ARF inhibition of E2F1 transcriptional activity (5, 42). Up-regulation of ARF by HDM2 suggests feedback regulation (70). Stabilization of HDM2 E3 ubiquitin ligase by MAGE-A11 facilitates increased E2F1 transcriptional activity by HDM2 and interferes with ARF inhibition of HDM2, which favors cell cycle progression by promoting p53 degradation. Interaction between MAGE-A11 and p300 inhibited MAGE-A11 stabilization of HDM2. MAGE-A11 is an integral player in transcriptional regulation required for cell cycle progression.

cycle progression (53–55). Our results suggest that MAGE-A11 is integral to a protein network involved in E2F1 regulation of cell cycle progression. The ability of MAGE-A11 to increase E2F1 transcriptional activity supports a critical role in prostate cancer cell growth.

A principal function of the ARF tumor suppressor is inhibition of HDM2, which results in the stabilization of p53 and loss of inhibition of E2F1. Both ARF and p53 tumor suppressor activity protect normal cells from oncogene-induced tumor formation (76). Previous studies suggested that ARF negatively regulates E2F1 through increased E2F1 ubiquitination and proteasome degradation (41, 53, 77). It was suggested that inhibition of E2F1-dependent transcriptional activity involves HDM2 and/or p53 (42, 78). We found that one function of ARF is inhibition of MAGE-A11 interaction with E2F1, which interferes with the stabilizing effects and transcriptional enhancing effects of MAGE-A11. Negative feedback regulation is suggested by the up-regulation of ARF and p53 by E2F1 that contributes to the induction of apoptosis (79, 80). ARF also interacts with c-myc and causes c-myc relocalization from the nucleoplasm to nucleoli, inhibits c-myc transcriptional activation of genes required for cell cycle progression (81), and induces sumoylation of its binding partners (57).

It remains to be determined what role MAGE-A11 has in the regulation of p53 and whether stabilization of HDM2 by MAGE-A11 interferes with ARF inhibition of HDM2 E3 ubiquitin ligase activity that promotes p53 degradation and cell cycle progression. It is not known how MAGE-A11 impacts feedback regulation of p53, such as the increase in ARF expression when p53 is inhibited or increased expression of ARF and p53 in response to cellular and viral oncogenes such as c-myc or E1A (82). Stabilization and sequestration of MAGE-A11 and

HDM2 by ARF may be involved in feedback control (79). Furthermore, HDM2 interaction with p300 is required for the proteasomal degradation of p53 (75). MAGE-A11 forms a strong complex with p300 in association with gene transcription (10) that inhibited the stabilization of HDM2 by MAGE-A11. These findings suggest that MAGE-A11 interaction with HDM2 and p300 may be involved in HDM2 regulation of p53. Phosphorylation of HDM2 by Akt signals the nuclear localization of HDM2. ARF inhibits nuclear export of HDM2 by the sequestration of HDM2 in nucleoli, where HDM2 no longer can export p53 to the cytoplasm for degradation and thereby activates p53 (24, 50, 58, 83– 85). The impact of MAGE-A11 on ARF and HDM2 regulation of p53 remains to be determined.

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