The ARF tumor suppressor targets PPM1G/PP2Cγ to counteract NF-κB transcription tuning cell survival and the inflammatory response

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Inducible transcriptional programs mediate the regulation of key biological processes and organismal functions. Despite their complexity, cells have evolved mechanisms to precisely control gene programs in response to environmental cues to regulate cell fate and maintain normal homeostasis. Upon stimulation with proinflammatory cytokines such as tumor necrosis factor-α (TNF), the master transcriptional regulator nuclear factor (NF)-κB utilizes the PPM1G/PP2Cγ phosphatase as a coactivator to normally induce inflammatory and cell survival programs. However, how PPM1G activity is precisely regulated to control NF-κB transcription magnitude and kinetics remains unknown. Here, we describe a mechanism by which the ARF tumor suppressor binds PPM1G to negatively regulate its coactivator function in the NF-κB circuit thereby promoting insult resolution. ARF becomes stabilized upon binding to PPM1G and forms a ternary protein complex with PPM1G and NF-κB at target gene promoters in a stimuli-dependent manner to provide tunable control of the NF-κB transcriptional program. Consistently, loss of ARF in colon epithelial cells leads to up-regulation of NF-κB antiapoptotic genes upon TNF stimulation and renders cells partially resistant to TNF-induced apoptosis in the presence of agents blocking the antiapoptotic program. Notably, patient tumor data analysis validates these findings by revealing that loss of *ARF* strongly correlates with sustained expression of inflammatory and cell survival programs. Collectively, we propose that PPM1G emerges as a therapeutic target in a variety of cancers arising from ARF epigenetic silencing, to loss of ARF function, as well as tumors bearing oncogenic NF-κB activation.

gene regulation | ARF | PPM1G | inflammatory response | NF-κB

Metazoan cells have evolved precise strategies to cope with cell-intrinsic and cell-extrinsic insults that impact tissue homeostasis (1). The NF-κB signaling pathway is critical in the response to a plethora of environmental stimuli, such as proinflammatory cytokines and stress insults. Normal functioning of this pathway relies on both cytoplasmic and nuclear events. In the cytoplasm, NF-κB exists in an inhibited state in which it is sequestered by a family of inhibitor of NF-κB (IκB) proteins (2). Exposure to one of multiple induction stimuli results in the phosphorylation of the IκB proteins by the IκB kinase (IKK) complex, subsequent ubiquitination, proteasomal degradation of the IκBs inhibiting NF-κB, and concomitant translocation of NF-κB to the nucleus, where it modulates gene transcription to regulate key biological processes, such as inflammation, cell survival, and apoptosis (3–7). This nuclear function of NF-κB as a master transcriptional regulator is what ultimately determines its role as either an oncogene or tumor suppressor in a diverse set of biological contexts (7–11).

In the nucleus, NF-κB functions along with transcriptional coactivators to fine tune RNA polymerase (Pol) II activity at different steps of the transcriptional cycle, such as initiation and elongation, in order to facilitate proper kinetic pulses of stimulus-dependent gene regulation (12, 13). Among the several coactivators identified to date, kinases, phosphatases, histone acetyltransferases (HATs), and deacetylases (HDACs) are the most commonly used to regulate transcription (14). These coactivators function by targeting NF-κB directly or by impinging at other levels in the transcriptional cycle, such as modifying the epigenetic landscape.

We have previously identified that the nuclear PPM1G/PP2Cγ phosphatase (one member of a family of metal-dependent Ser/Thr phosphatases) (15) is a NF-κB transcriptional coactivator (16–18). Upon stimulation and NF-κB nuclear localization, the RelA subunit of the NF-κB family directly binds to PPM1G and selectively recruits it to NF-κB target gene promoters to activate the transition between initiation and elongation, an essential step for gene activation (19–21). Several RelA/NF-κB target genes rely on P-TEFb (dimer of the CDK9 kinase and its cyclin subunit [T1/T2]) to stimulate NF-κB–mediated transcription elongation (7). PPM1G regulates the activity of P-TEFb by dephosphorylating the activating T loop of CDK9, releasing P-TEFb from its inactive form (bound to the 7SK small nuclear ribonucleoprotein complex [snRNP]) to its active form (7SK-unbound P-TEFb), which subsequently enables productive transcription elongation of NF-κB target genes (16, 20, 22, 23). Given that NF-κB transcription of antiapoptotic genes in response to inflammatory

Significance

The NF-κB signaling pathway is critical for cellular responses to cell-extrinsic insults. Its dysregulation impacts cancer patients' survival and response to available therapeutics. NF-κB utilizes the PPM1G phosphatase as a coactivator to induce inflammatory and cell survival programs in response to proinflammatory cytokines. However, how PPM1G activity is regulated to facilitate tunable control and insult resolution remains enigmatic. We take advantage of biochemical and genetic approaches and patient tumor data analysis to reveal a key function of the $p14^{ARF}$ tumor suppressor in targeting PPM1G to negatively tune NF-κB transcriptional function, thereby controlling inflammatory and apoptotic responses. We propose that PPM1G emerges as a therapeutic target in a variety of cancers arising from ARF loss of function to oncogenic NF-κB activation.

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The authors declare no competing interest.

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cytokines is crucial for cell survival, loss of PPM1G induces cell sensitivity to TNF-α (hereafter referred to as TNF)-mediated apoptosis (8, 16, 18, 24). Thus, PPM1G is a critical transcriptional coactivator that fulfills essential functions to properly regulate NF-κB activity and cellular responses.

In efforts to elucidate molecular mechanisms involved in the regulation of NF-κB activity through PPM1G, we implemented an unbiased affinity purification and mass spectrometry (AP-MS) screening approach and identified the $p14^{ARF}$ tumor suppressor (*CDKN2A*/*INK4A* locus), hereafter referred to as ARF, as one of the top interacting proteins. We found that ARF directly associates with PPM1G to negatively regulate NF-κB transcription in response to inflammatory stimuli to tune NF-κB transcriptional responses and promote TNF-induced apoptosis. Importantly, patient data analysis revealed that tumors lacking *ARF*, as frequently seen in several types of cancer (e.g., sarcoma, glioma, and T cell lymphoma) (25), are associated with sustained expression of inflammatory and antiapoptotic programs through potential deregulation of NF-κB transcription program deactivation. We discuss the implications of our discoveries for the roles of NF-κB and ARF in human cancer and possible therapeutic interventions.

Results

Unbiased Screening for PPM1G Interactors Identifies the ARF Tumor Suppressor. We have previously described that PPM1G functions as a coactivator of NF-κB transcription in response to inflammatory and genotoxic stimulation (16, 18). To unbiasedly identify PPM1G interacting proteins that could override NF-κB activity and facilitate insult resolution, we expressed Strep-tagged PPM1G in HEK293T cells and performed Strep affinity purification (AP) coupled with mass spectrometry (MS) analysis (Fig. 1*A*). Interestingly, we found that PPM1G associates with ARF (known as $p14^{ARF}$ in humans and $p19^{ARF}$ in mouse) and $p16^{INK4a}$ (Fig. 1*B*), two tumor suppressor products of the INK4a/ARF (*CDKN2A*) locus (26). Notably, we also found a high-confidence interaction with the histone H2A-H2B dimer, which was previously described to interact with PPM1G (27), as well as an undescribed binding partner, Myb-binding protein 1A (MYBBP1A) (Fig. 1*B* and Dataset S1). Strikingly, ARF was previously described to modulate the NF-κB transcriptional program (28–30), suggesting a potentially uncharacterized functional interplay between ARF and PPM1G in the control of NF-κB activity.

To validate the MS results, we engineered epitope-tagged constructs for protein–protein interaction assays. We coexpressed Strep-tagged PPM1G and Flag-tagged ARF in HEK293T cells, and after Strep AP, observed that ARF indeed copurifies with PPM1G (Fig. 1*C*). The reciprocal experiment also showed that Strep-tagged ARF copurifies with Flag-tagged PPM1G (Fig. 1*D*), demonstrating that the PPM1G–ARF protein interaction is specific, epitope independent, and that both proteins copurify irrespective of the order of purification. Importantly, purification of Strep-tagged PPM1G revealed an interaction with endogenous ARF (Fig. 1*E*) and indirect immunofluorescence showed colocalization between endogenous PPM1G and Flag-tagged ARF in the nucleus of living cells (Fig. 1*F*). To test whether the interaction is direct, we coexpressed His-tagged PPM1G and Strep-tagged ARF (or only the individual components) in a heterologous bacterial system and performed nickel-nitrilotriacetic acid (Ni-NTA) AP and then analyzed for protein–protein interactions. We observed that His-tagged PPM1G copurified with Strep-tagged ARF, providing supporting evidence that the PPM1G–ARF interaction is direct (Fig. 1*G*).

Together, these data provide compelling evidence that ARF and PPM1G physically interact in vitro and in cells. Given that PPM1G is a transcriptional coactivator of NF-κB and that ARF regulates NF-κB activity (16, 18, 28), we describe below how we performed biochemical dissection of the interactions, investigated the roles of the PPM1G–ARF complex in NF-κB transcriptional responses, examined cellular consequences, and finally we provide clinical relevance.

The PPM1G C-Terminal Domain Is Required for ARF Binding and Stabilization, and PPM1G Bridges ARF and NF-κB to Assemble a Ternary Complex. To dissect out the molecular basis of the PPM1G–ARF protein–protein interaction, we performed domain-mapping analysis using interaction assays in HEK293T cells (Fig. 2). We coexpressed Strep-tagged ARF and Flag-tagged full-length (FL) PPM1G or individual domains (N terminal, acidic, and C terminal) followed by a Strep AP to purify ARF, and then

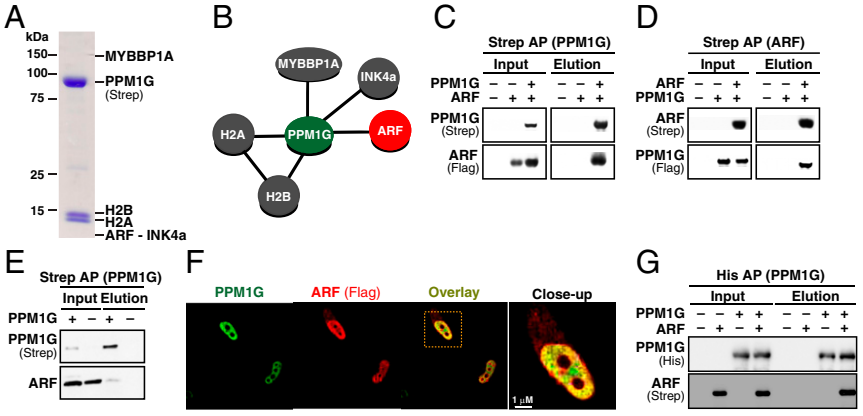

Fig. 1. Unbiased screening for PPM1G interactors identifies the ARF tumor suppressor. (*A*) Strep AP of PPM1G from HEK293T cells. High-confidence interactors were identified by MS analysis (Dataset S1). The positions of the identified interactors (ARF and MYBBP1A) are indicated with lines, but not detected by Coomassie staining due to sensitivity. (*B*) Network representation of high confidence PPM1G interactors with protein coverage greater than 30%. A complete list of all interactors and their semiquantitative abundance is included in the Dataset S1. (*C*) Validation of the PPM1G–ARF interaction. Flag-tagged ARF was expressed into cells along with either Strep-tagged PPM1G or empty plasmid (–). Strep AP was performed followed by Western blot with the indicated antibodies. (*D*) Reciprocal validation of the PPM1G–ARF interaction. Flag-tagged PPM1G was expressed into cells along with Strep-tagged ARF or empty plasmid (–). Strep AP was performed followed by Western blot with the indicated antibodies. (*E*) Further validation of the PPM1G–ARF interaction. Strep-tagged PPM1G or empty plasmid (–) were expressed into cells and Strep AP was performed followed by Western blot of ectopically expressed PPM1G and endogenous ARF. (*F*) PPM1G and ARF colocalize in the nuclei of living cells. HeLa cells were used for indirect immunofluorescence. Confocal images were acquired with a closeup view (3× magnification) of a single cell. (Scale bar, 1 μM.) (*G*) PPM1G and ARF directly bind in vitro. His-tagged PPM1G and Strep-tagged ARF were expressed individually or coexpressed in bacterial cells. A Ni-NTA purification was performed followed by Western blot with the indicated antibodies. Data in all panels are representative of three independent experiments.

we assessed PPM1G interactions. We found that ARF binds to the C-terminal domain of PPM1G, but not to its N-terminal or acidic domains (Fig. 2*A*). Supporting this initial observation, deletion of the C-terminal domain (PPM1GΔC) virtually abolished ARF binding (Fig. 2*B*), indicating that the C-terminal domain of PPM1G is necessary and sufficient for ARF recognition. Strikingly, we also noticed that PPM1G expression appeared to stabilize ARF in both whole cell lysates (inputs) and after Strep AP when compared to relative ARF levels in cells lacking PPM1G or only expressing the individual domains (Fig. 2 *A* and *B*). To further test this idea, we ectopically expressed Strep-tagged ARF with increasing amounts of Flag-tagged PPM1G or negative controls that do not bind ARF (PPM1GΔC and GFP), and performed Western blots on total cell lysates. Consistent with our previous observations, expression of PPM1G, but not PPM1GΔC or GFP, promoted ARF stabilization in a dose-dependent manner (Fig. 2*C*).

Given that ARF binding to the PPM1G C-terminal domain alone does not appear to directly correlate with ARF stabilization (Fig. 2*A*), we reasoned that PPM1G's catalytic activity might be required to stabilize ARF upon binding. To test this idea, we coexpressed Strep-tagged ARF with Flag-tagged wild-type (WT) PPM1G or a previously characterized, catalytically inactive (D496A) PPM1G mutant (named MUT) (16), and observed that both bind and stabilize ARF equally well (~1.14-fold difference in ARF levels when comparing PPM1G WT vs. MUT-expressing cells, Fig. 2*D*). This suggests that PPM1G's phosphatase activity is dispensable for ARF stabilization and that PPM1G may act through other mechanisms such as protecting ARF from rapid protein turnover due to ubiquitination and proteasome-mediated degradation (31).

Because both ARF and NF-κB bind PPM1G, possibly with opposing functional outputs (16, 28), we asked whether they compete for association to PPM1G by performing domain-mapping analysis of the PPM1G–ARF and PPM1G–NF-κB interactions. To define how PPM1G binds ARF, we coexpressed Strep-tagged PPM1G and either Flag-tagged full-length ARF or its individual domains (N and C terminal) followed by Strep AP. We found that PPM1G binds to the N-terminal domain of ARF (*SI Appendix*, Fig. S1*A*), a target site previously implicated in binding the p53-ubiquitin ligase MDM2/HDM2 (32), suggesting that the ARF–PPM1G complex might function in a MDM2- and p53-independent manner (see below). In addition, the N terminal of ARF can be polyubiquitinated to promote ARF degradation (31), potentially explaining why PPM1G binding to the N-terminal domain leads to ARF stabilization.

To examine how NF-κB (RelA subunit) associates with PPM1G, we coexpressed Flag-tagged PPM1G or its individual domains and HA-tagged NF-κB followed by Flag immunoprecipitation (IP). Interestingly, we found that NF-κB contacts both the N- and C-terminal regions in PPM1G, but not its acidic domain (*SI Appendix*, Fig. S1*B*). To dissect out how PPM1G binds NF-κB, we coexpressed Flag-tagged PPM1G and HA-tagged full-length NF-κB or its individual domains (rel homology domain [RHD] and transactivation domain [TAD]), and found that PPM1G selectively contacted the RHD, but not the TAD (*SI Appendix*, Fig. S1*C*). Interestingly, the RHD domain contains an immunoglobulin-like fold (PF16179) that mediates many other protein–protein interactions, thus supporting the biochemical data.

Because both ARF and NF-κB bind the PPM1G C-terminal domain, we asked whether they compete for PPM1G association ("competitive" model) or form a larger complex ("ternary-complex" model) (*SI Appendix*, Fig. S1*D*) that could modulate or interfere with PPM1G's functions. To distinguish between these two models, we cotransfected ARF, PPM1G, and NF-κB expressing plasmids with different epitopes (PPM1G:S, ARF:F, and NF-κB:HA) and performed biochemical purifications. Since an AP

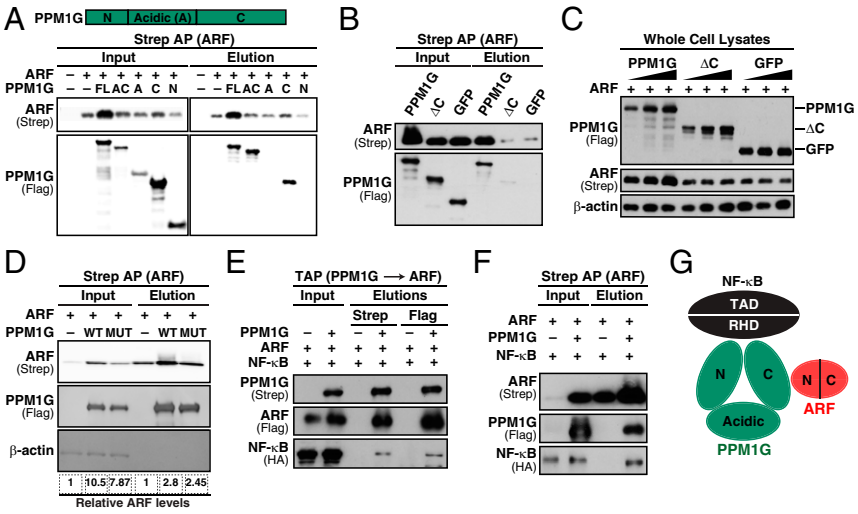

Fig. 2. PPM1G stabilizes ARF and bridges ARF and NF-κB to assemble a ternary protein complex. (*A*) ARF binds to the PPM1G C-terminal domain. Strep-tagged ARF and Flag-tagged FL PPM1G or its individual domains were cotransfected into HEK293T cells. Strep AP was performed followed by Western blot with the indicated antibodies. (*B*) The C-terminal domain of PPM1G is required for ARF binding. Strep-tagged ARF along with Flag-tagged PPM1G, PPM1GΔC, or GFP were cotransfected into cells. Strep AP was performed followed by Western blot with the indicated antibodies. (*C*) The C-terminal domain of PPM1G is required for ARF stabilization. Strep-tagged ARF along with Flag-tagged PPM1G, PPM1GΔC, or GFP plasmids were cotransfected into cells. Protein lysates were then analyzed by Western blot with the indicated antibodies. (*D*) The catalytic activity of PPM1G is not required to promote ARF stabilization upon ARF binding. Cells were cotransfected with Strep-tagged ARF along with Flag-tagged wild-type (WT) PPM1G, the catalytically inactive PPM1G mutant D496A (MUT) or empty plasmid (–). Strep AP was performed followed by Western blot with the indicated antibodies. (*E*) ARF forms a ternary protein complex with PPM1G and NF-κB. Flag-tagged ARF and HA-tagged NF-κB were cotransfected in cells in the presence/absence of Strep-tagged PPM1G, and a TAP was performed (Strep AP followed by Flag IP) and analyzed by Western blot with the indicated antibodies. (*F*) ARF requires PPM1G to bridge the interaction with NF-κB. Strep-tagged ARF and HA-tagged NF-κB were cotransfected in the presence or absence of Flag-tagged PPM1G. Strep AP was performed followed by Western blot with the indicated antibodies. (*G*) Simplified model of the domain interaction in the ARF–PPM1G–NF-κB protein complex. PPM1G mediates the ARF–NF-κB interaction by interacting with the RHD of NF-κB and the N terminal of ARF. Data in all panels are representative of three independent experiments.

step could not distinguish between the formation of independent protein complexes (PPM1G–NF-κB and PPM1G–ARF) or ternary protein complexes in a mutually exclusive manner, we applied a tandem affinity purification (TAP) protocol described by our group (33). TAP allows for the subsequent purification of protein complexes through consecutive purification steps (Strep and Flag) followed by Western blot analysis. Notably, we observed evidence of an interaction between ARF, PPM1G, and NF-κB in both the first (Strep) and second (Flag) elutions of samples containing Strep-tagged PPM1G, but not in the negative control lacking PPM1G, providing evidence of ternary complex formation (Fig. 2*E*).

To cross-validate these data, we exchanged the epitope tags between PPM1G and ARF, cotransfected NF-κB, and again performed a TAP. Importantly, we found that NF-κB was present in the TAP of PPM1G–ARF complexes, but not in the negative control (no ARF) (*SI Appendix*, Fig. S1*E*), thereby strengthening the ternary complex model (*SI Appendix*, Fig. S1*D*). In addition, we observed that NF-κB is recovered from ARF purifications only if PPM1G is present, indicating that PPM1G is the bridge that links ARF and NF-κB for protein complex assembly (Fig. 2*F*).

Taken together, using the domain mapping information, we propose a simplified model that depicts the potential assembly of the ARF–PPM1G–NF-κB ternary complex, where PPM1G functions as a scaffold linking ARF through its N-terminal domain and NF-κB through its RHD module (Fig. 2*G*). Below we investigate the functional significance of this protein complex and its physiological relevance for the NF-κB transcriptional program in directing cell fate decisions.

ARF Expression in an ARF-Minus Background Blocks TNF-Induced, PPM1G-Dependent NF-κB Transcriptional Activity and Triggers TNF-Dependent Cell Death. To gain insights into whether the ARF–PPM1G–NF-κB ternary complex is functional in the TNF-mediated NF-κB transcriptional program, we used an isogenic, p53-minus cell system called NARF2-E6 (28), a derivative of NARF2 cells (34), which are osteosarcoma (ARF-negative) cells ectopically expressing ARF in an isopropyl β-D-1 thiogalactopyranoside (IPTG)-dependent manner (Fig. 3*A*) and in which NF-κB activation is PPM1G dependent (*SI Appendix*, Fig. S2 *A* and *B*).

To establish the experimental system to test the influence of ARF in the NF-κB transcriptional program and cellular functional consequences, we treated NARF2-E6 cells with IPTG (or vehicle control) to induce ARF expression (Fig. 3*A*) followed by TNF stimulation (or vehicle control) at different time points (2, 8, and 24 h) (Fig. 3*B*). We then measured kinetics of NF-κB target gene expression and control genes by RT-qPCR (Fig. 3 *C–F* and *SI Appendix*, Fig. S2 *C–H*) and cell apoptosis by fluorescence-activated cell sorting (FACS) (Fig. 3*G*). We tested several NF-κB targets from a variety of families to determine if there was any specificity, including proinflammatory genes like *IL8* and *TNFα* (Fig. 3 *C* and *D*), antiinflammatory genes like *TNFAIP3* and *NFKBIA* (Fig. 3*E* and *SI Appendix*, Fig. S2*C*), antiapoptotic genes from the XIAP (*BIRC3*, *XIAP*, and *BIRC2*), *Bcl-XL* (*BCL2L1*), and FLIP (*cFLAR*) families, as well as *GAPDH* as a negative control (Fig. 3*F* and *SI Appendix*, Fig. S2 *D–H*). Interestingly, we observed a significant reduction in the expression of TNF-stimulated genes, including inflammatory (*IL8* and *TNFα*), negative regulators (*NFKBIA* and *TNFAIP3*), and antiapoptotic (*BIRC3*, *BCL2L1*, *XIAP*, and *BIRC2*), with no changes in the non–NF-κB target gene (*GAPDH*) in the presence of ARF, suggesting that ARF specifically regulates NF-κB transcription activity in response to TNF stimulation. While most genes had reduced expression across all time points tested, some genes (*NFKBIA* and *BCL2L1*) had a delayed response to TNF in the presence of ARF where gene expression increased at a later time point (8 or 24 h). Unexpectedly, expression of *cFLAR* upon TNF induction remained mostly unaffected by ARF (*SI Appendix*, Fig. S2*G*), suggesting that ARF may not regulate, at least in this cell type, the FLIP family of NF-κB targets genes.

Given these findings, we then wondered if decreased expression of antiapoptotic genes in response to TNF upon ARF expression resulted in an enhanced sensitivity to apoptosis. Strikingly, while TNF alone did not induce significant cell death as expected, ARF expression in the presence of TNF significantly decreased cell survival (revealed as a temporal increase in annexin V-positive cells) as early as 8 h post-TNF stimulation (Fig. 3*G*), consistent with previous results (28), thereby complementing the robust gene expression defects observed upon ARF expression (Fig. 3 *C–F*). Interestingly, this observation upon ARF induction phenocopies how loss of PPM1G triggers sensitivity to TNF-dependent cell death (8, 16, 18, 24). Together, these data reveal that ARF has repressive functions in the inflammatory response and that ARF ectopic expression triggers TNF-induced cell death, which may be explained, at least in part, by ARF dampening PPM1G-mediated NF-κB transcription (see below).

TNF Induces Formation of the ARF–PPM1G–NF-κB Ternary Complex and Its Occupancy at Target Gene Promoters. Given that ARF assembles a ternary complex with PPM1G and NF-κB (Fig. 2), and because ARF expression blocks NF-κB transcription in response to TNF stimulation (Fig. 3), we asked whether TNF stimulates the formation of the ternary complex. To test this idea, we performed a single Strep AP of ectopically expressed PPM1G from HEK293T cells treated in the absence and presence of TNF, and in crosslinked conditions to eliminate possible in vitro reassortments of protein–protein interactions not occurring in cells during cell lysis and protein purification (Fig. 4*A*). We observed that while the PPM1G–NF-κB interaction was largely (~11-fold) induced by TNF, most likely due to cytoplasmic–nuclear translocation of NF-κB upon stimulation (16), the basal level of PPM1G–ARF interactions in the absence of treatment remained unchanged after stimulation (Fig. 4*A*).

Because NF-κB activity is tightly regulated by posttranslational modifications (PTMs) that facilitate or prevent cofactor interactions, we assessed the status of NF-κB in the ternary complex by probing for site-specific phosphorylation (P-S536), an activating PTM that promotes CBP/p300 binding, and is induced early upon stimulation (35). Interestingly, we found that PPM1G binds endogenous, phosphorylated NF-κB only in response to TNF stimulation (Fig. 4*A*), in agreement with the increased PPM1G–NF-κB interactions upon stimulation.

To further assess the inducibility of the ternary complex upon stimulation, and because a single AP does not truly allow for interrogating interactions of the three subunits as part of the ternary complex, we performed a TAP (PPM1G:S followed by ARF:F) from cells treated with TNF (or vehicle control) and in cross-linked conditions, and found that the PPM1G–ARF complex pulled down a larger fraction (~sevenfold) of NF-κB:HA (and phosphorylated NF-κB) in TNF-treated cells (Fig. 4*B*), cross-validating the single AP experiment (Fig. 4*A*) and strengthening the model that TNF induces formation of the ternary complex, in which NF-κB is phosphorylated. While in the TAP of ectopically expressed components there is some phosphorylated NF-κB in the elution in unstimulated conditions that is not detectable in the input (Fig. 4*B*), this may be attributed to ectopic expression of NF-κB, which may force abnormal partial nuclear localization and phosphorylation of NF-κB.

Given the above results of TNF-mediated ternary complex formation, we then asked whether ARF functions with PPM1G and NF-κB on chromatin to regulate target gene expression. To test this idea, ARF was induced (or not) in NARF2-E6 cells treated with TNF (or vehicle control) for 2 h, followed by nuclei isolation for chromatin immunoprecipitation (ChIP) assays with antibodies against NF-κB (RelA subunit), PPM1G, and ARF, and qPCR analysis to assess factor enrichment on NF-κB target

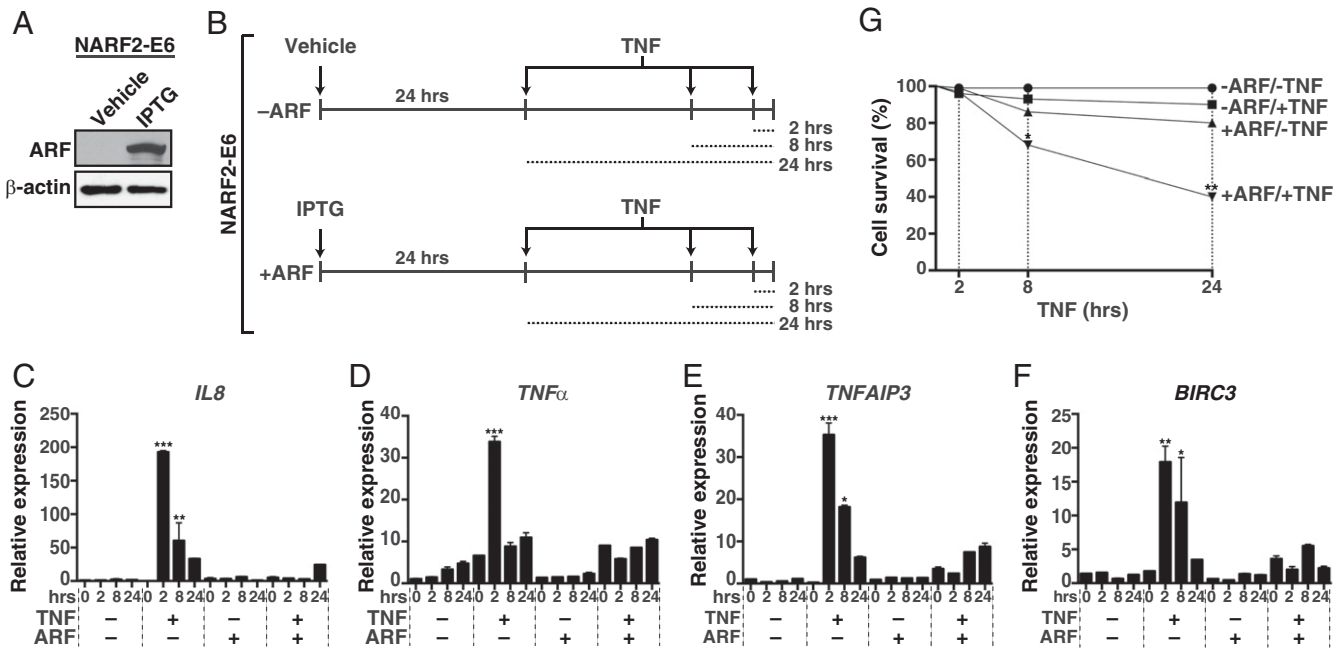

Fig. 3. ARF expression in an ARF-minus background dampens TNF-induced NF-κB transcription and promotes TNF-dependent cell death. (*A*) Western blot showing ARF induction in NARF2-E6 cells in response to a 48-h IPTG treatment. (*B*) Scheme of the protocol used to induce ARF with IPTG or not (vehicle control) from NARF2-E6 cells with TNF treatment at different time points post-ARF induction. (*C–F*) ARF blocks TNF-induced NF-κB transcription. NARF2-E6 cells were used to induce ARF or not and then treated in the presence or absence of TNF for the indicated time points. The expression of NF-κB target genes was measured by RT-qPCR and normalized to β-actin (mean ± SEM are shown; $n = 3$). (*G*) ARF triggers TNF-dependent cell death. NARF2-E6 cells were treated as indicated in *B*. Cells were collected for annexin V and propidium iodide (PI) staining and % annexin V-positive cells (Cell survival) were plotted in response to either noninduction or induction of ARF and in the presence or absence of TNF treatment. Statistical significance between the indicated pairwise comparisons was calculated using unpaired Student's *t* test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. For *C–G*, the asterisks represent the statistical significance between samples –ARF+TNF and +ARF+TNF for the same time point.

genes: *IL8* (Fig. 4*C*) and *BIRC3* (*SI Appendix*, Fig. S3*A*). Notably, we found evidence of ARF, PPM1G, and NF-κB co-occupancy at the *IL8* (Fig. 4*C*) and *BIRC3* (*SI Appendix*, Fig. S3*A*) promoters in response to TNF stimulation, but no occupancy at the *GAPDH* promoter (*SI Appendix*, Fig. S3*B*), which was used as a negative gene control because its expression is not altered by TNF treatment nor by ARF expression (*SI Appendix*, Fig. S2*H*). Remarkably, these results are consistent with biochemical evidence that TNF induces formation of the ARF–PPM1G–NF-κB ternary protein complex and that ARF interferes with PPM1G coactivator function in the NF-κB transcriptional program (Figs. 2–4).

Despite the observed co-occupancy of ARF–PPM1G–NF-κB ternary complex at the *IL8* (and *BIRC3*) promoter-proximal regions in response to TNF stimulation, we found no recruitment of ARF, PPM1G, and NF-κB to the gene bodies (promoter-distal regions) (Fig. 4*C* and *SI Appendix*, Fig. S3*A*), suggesting that ARF regulates PPM1G–NF-κB function at the promoter level. Interestingly, while ARF dampened expression of *IL8* and *BIRC3* in response to stimulation, NF-κB recruitment was only slightly reduced at the *IL8* (~1.5-fold) (Fig. 4*C*) and *BIRC3* (~1.2-fold) gene promoters (*SI Appendix*, Fig. S3*A*), indicating that the primary function of ARF is not to compete with NF-κB for binding to its target sites on gene promoters, as has been reported for other factors (36), consistent with the lack of ARF competition for NF-κB binding to PPM1G (Fig. 2 and *SI Appendix*, Fig. S1).

Given that PPM1G promotes Pol II pause release and elongation (16–18), we wondered if ARF–PPM1G interactions at NF-κB target genes upon TNF stimulation can influence Pol II pause release and/or other earlier steps in the transcription cycle such as Pol II promoter recruitment and/or initiation. To test this idea, we used ChIP assays to monitor levels of Pol II at promoter-proximal and promoter-distal regions indicative of initiation and elongation, respectively. Interestingly, we found that upon ARF induction, Pol II levels decreased at both regions in the *IL8* locus, but with a larger effect at the promoter-distal position (~fourfold) compared to the promoter-proximal region (~twofold). This result signifies that by occupying NF-κB target gene promoters with NF-κB and its coactivator PPM1G (and potentially assembling a ternary complex (Figs. 2 and 4 *A* and *B*), ARF dampens PPM1G-dependent transcription elongation, which restricts the Pol II pause release step (Fig. 4*C*) (16–18). Similar results were observed at the *BIRC3* locus (*SI Appendix*, Fig. S3*A*) with no occupancy changes at either region in *GAPDH* (*SI Appendix*, Fig. S3*B*), strongly indicating that the observed mechanism is specific for NF-κB target genes.

Collectively, these data provide evidence that the ARF tumor suppressor has the ability to tune Pol II transcription in response to proinflammatory stimulation by assembling and potentially blocking the function of elongation factors (PPM1G) at select NF-κB target genes thereby leading to intimate control of the inflammatory response and dampening of cell survival. Although this evidence derives from the use of a simple system in which ARF is artificially induced with exogenous treatment, it enabled us to define an unprecedented regulatory mechanism of ARF function in the NF-κB transcriptional program and is consistent with the biochemical data.

Loss of ARF in Colon Epithelial Cells Leads to Compromised NF-κB Insult Resolution and Resistance to TNF-Induced Apoptosis in the Presence of Agents that Block the Antiapoptotic Program. Because the previous data were collected in a system in which ARF expression was artificially induced, we wanted to test if ARF control of PPM1G-dependent NF-κB function has any physiological relevance.

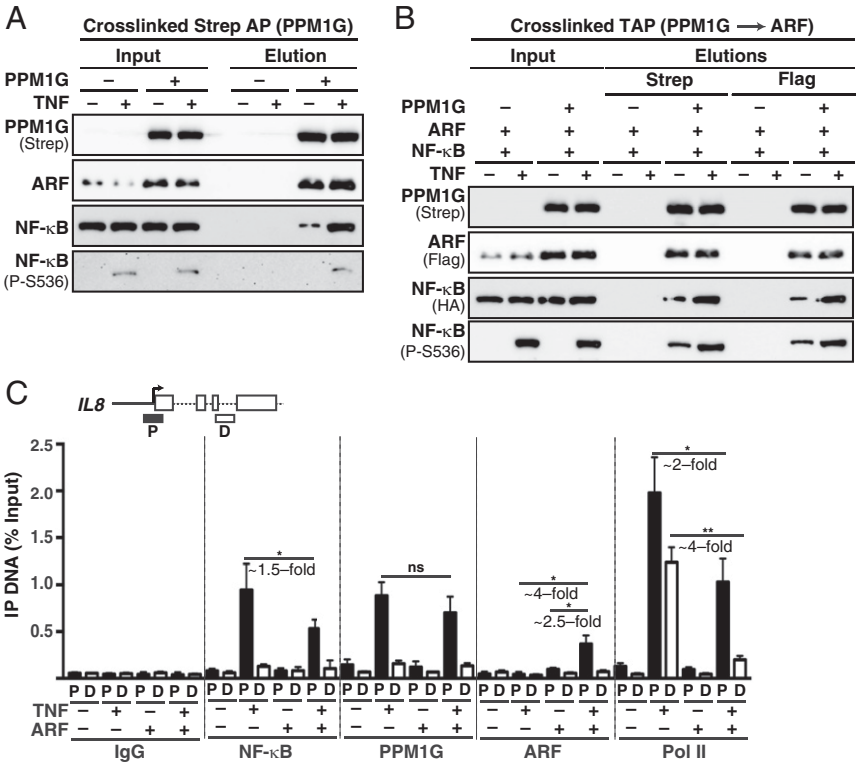

Fig. 4. TNF induces the PPM1G–ARF–NF-κB ternary complex on chromatin. (*A*) The PPM1G–ARF interaction is independent of TNF. HEK293T cells were transfected with Strep-tagged PPM1G or empty plasmid (–), cells were treated with or without TNF, cross-linked, and lysed under denaturing conditions. Strep AP was performed to analyze the interaction between PPM1G, ARF, and NF-κB (including NF-κB P-S536). (*B*) TNF induces ternary complex formation. Flag-tagged ARF and HA-tagged NF-κB were cotransfected in cells in the presence/absence of Strep-tagged PPM1G, cells were treated with or without TNF, cross-linked, and lysed under denaturing conditions. TAP was performed (Strep AP followed by Flag IP) and analyzed by Western blot with the indicated antibodies. (*C*) ARF binds NF-κB target gene promoters in response to TNF treatment alongside with NF-κB and PPM1G to block Pol II transcription. (*Top*) Scheme of the *IL8* loci with the positions of the amplicons used in ChIP-qPCR assays (P, promoter-proximal; D, promoter-distal). (*Bottom*) ChIP assays were performed with protein extracts from four treatment conditions and the indicated antibodies followed by qPCR with the P and D amplicons to monitor factor interactions on the target locus. A normal IgG serum was used as a negative control in ChIP-qPCR. The ChIP-qPCR data were normalized using the "percent input method" (see *Materials and Methods* for full description). Values represent the percentage (%) of input DNA immunoprecipitated (IP DNA) and are the average of three independent experiments (mean ± SEM; $n = 3$). Statistical significance between the indicated pairwise comparisons was calculated using unpaired Student's *t* test: $*P < 0.05$, $**P < 0.01$, ns, non-significant.

In hopes of identifying a system in which we could assess functional consequences upon ARF loss of function, we first searched for tissues and cell types in which ARF might be expressed. According to data from the Human Protein Atlas, ARF is expressed in various tissues in normal conditions, including the gastrointestinal tract (https://www.proteinatlas.org/ENSG00000147889-CDKN2A/tissue), and for this reason we turned to an immortalized, primary human colonic epithelial cell model system (HCEC) (37). After validating ARF expression in HCEC by Western blot, we acutely silenced ARF using RNAi-mediated knockdown (KD) with a siRNA only targeting $p14^{ARF}$ (but not $p16^{INK4A}$) (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S4*A*). Importantly, ARF silencing does not alter NF-κB and PPM1G expression, although $p16^{INK4A}$ levels were slightly (~1.5-fold) increased (*SI Appendix*, Fig. S4*A*), potentially due to a compensatory effect owing to ARF silencing. However, while this increase in p16 expression has been reported previously, $p16^{INK4A}$ is a negative regulator of the TNF response (38, 39), so increased p16 levels cannot explain the observed increased NF-κB transcriptional responses (see below).

Having established a system to study functional consequences upon ARF loss, we then treated both control (siNT) and ARF KD (siARF) HCEC with TNF for a time course (0, 4, 24, and 48 h) to examine whether loss of ARF indeed leads to enhanced expression of various NF-κB target genes in this physiological system using RT-qPCR assays (Fig. 5 *C*–*G* and *SI Appendix*, Fig. S4 *B*–*E*). Remarkably, we observed that ARF silencing increased the expression levels of proinflammatory genes like *IL8* and *IL1β*, at all time points examined (4, 24, and 48 h), suggesting an inflammatory signature (Fig. 5 *C* and *D*), in accordance with the decreased expression of proinflammatory genes upon ARF ectopic expression (Fig. 3). Additionally, the *CXCL10* chemokine also shows ~three- to eightfold increased expression at 4 to 24 h after TNF treatment in siARF cells, but then reaches almost normal levels by 48 h, potentially indicating that loss of ARF largely compromises the magnitude of expression of this chemokine at earlier time points (Fig. 5*E*). The antiapoptotic gene *BIRC3* and antiinflammatory gene *NFKBIA* also showed a similar pattern (Fig. 5 *F* and *G*). As expected, the observed phenotypes did not occur at non–NF-κB target genes such as 7SK (*SI Appendix*, Fig. S4*B*). Surprisingly, we found that not all NF-κB target genes (e.g., *TNFAIP3*, *BIRC2*, and *cFLAR*) appear to be significantly regulated by ARF (*SI Appendix*, Fig. S4 *C*–*E*), raising interesting questions of target gene specificity for future studies.

Given the transcriptional changes leading to both induction of antiapoptotic genes upon ARF loss in HCEC (Fig. 5) and block of antiapoptotic genes upon ARF ectopic expression in an ARF-minus context (Fig. 3), we predicted that loss of ARF may reduce sensitivity of cells to TNF-induced apoptosis. However, while TNF alone does not induce apoptosis because NF-κB activates transcription of antiapoptotic genes that counteract non–NF-κB–dependent activation of caspases that promote apoptosis (8, 24, 40–42), the presence of agents that can block the NF-κB

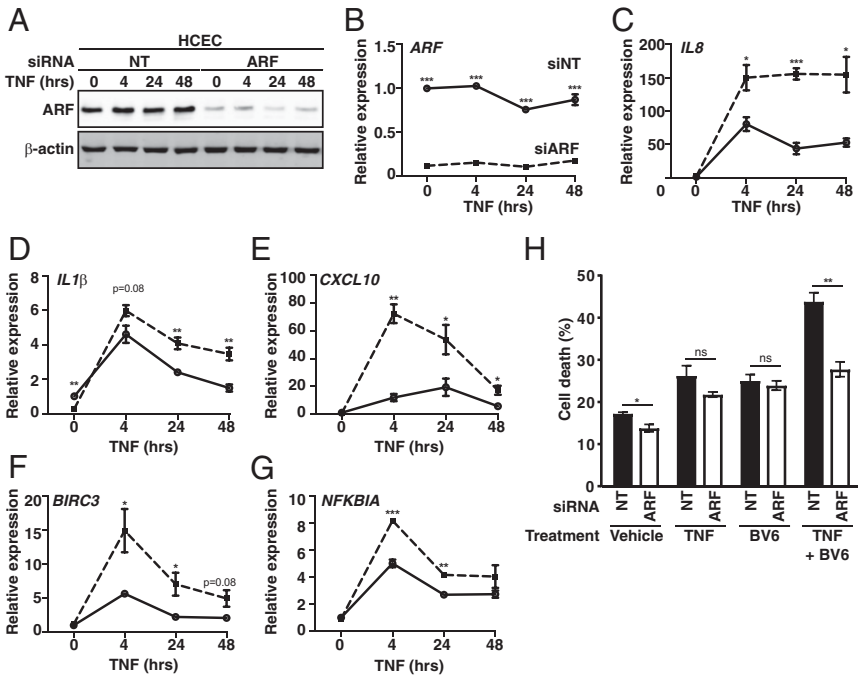

Fig. 5. Loss of ARF in colon epithelial cells leads to aberrant activation of antiapoptotic and inflammatory genes, leading cells to be resistant to TNF-induced apoptosis. (*A*) ARF expression was silenced in HCEC using siRNA and then analyzed by Western blot with the indicated antibodies. (*B*–*G*) ARF silencing leads to hyperactivation of NF-κB transcription activity in response to TNF stimulation. The expression of ARF and several NF-κB target genes (siNT, solid lines; siARF, dashed lines) were measured by RT-qPCR and normalized to U6 (mean ± SEM are shown; $n = 3$). (*H*) Loss of ARF renders cells resistant to TNF-induced apoptosis. siNT and siARF HCEC were treated with vehicle, TNF alone, a SMAC mimetic (BV6), and TNF + BV6 for 12 h. Cells were collected for annexin V and PI staining and % annexin V-positive cells were plotted. Statistical significance was calculated using unpaired Student's *t* test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ns, non-significant. The asterisks represent the statistical significance between samples for the same time point.

antiapoptotic response in cotreatment with TNF has been shown to trigger TNF-induced apoptosis (43). Thus, we leveraged this knowledge to test if ARF loss renders siARF cells resistant to TNF-induced apoptosis compared to siNT cells by cotreating HCEC with TNF and BV6, a SMAC mimetic (SMACm) that induces the autoubiquitination and degradation of antiapoptotic factors (BIRCs) thereby culminating in TNF-mediated cell death (44, 45). Interestingly, cotreatment with TNF and BV6, but not the individual drug treatments, enhanced apoptosis of siNT cells (~2.5-fold), whereas siARF cells were partially resistant as fewer cells were considered early apoptotic or dead (~1.58-fold decrease between siNT and siARF) as revealed by annexin V and PI staining followed by FACS (Fig. 5*H*).

Collectively, loss of ARF expression in HCEC causes partial resistance to TNF-induced apoptosis in the presence of SMACm, consistent with the significant increased expression of some antiapoptotic genes (Fig. 5 *A*–*G*). Notably, these data cross-validate the decreased expression of antiapoptotic products and increased apoptosis of NARF2-E6 cells upon ARF ectopic expression in response to TNF stimulation (Fig. 3).

ARF Fine Tunes TNF-Induced, PPM1G-Dependent NF-κB Transcription. The above results indicate that ARF controls NF-κB transcription activity, but do not prove PPM1G dependence. To determine whether ARF dampens TNF-induced NF-κB transcription in a PPM1G-dependent manner, we first needed to define how ARF binds to PPM1G to then create ARF mutants that can be tested in functional assays (results summarized in Fig. 6*A*). Given that the three-dimensional (3D) structure of ARF is unknown, we used in silico tools to predict the secondary structure of ARF and found that it putatively contains two parallel β-sheets and one α-helix within the N-terminal domain (residues 1 to 55) (*SI Appendix*, Fig. S5*A*). Guided by this predictive model, and because the first 64 residues of ARF were required for PPM1G binding (Fig. 2 and *SI Appendix*, Fig. S1*A*), we created several N-terminal deletions (12 to 132, 20 to 132, 29 to 132, 41 to 132, and 65 to 132) that were expressed in HEK293T cells for protein interaction assays. We observed that while deletion of the first 28 residues (29 to 132 construct) did not apparently influence PPM1G binding, deletion of the first 40 residues (41 to 132 construct) reduced binding by more than ~90% (labeled as -/+ PPM1G binding) and looked similar, if not identical, to the deletion of the first 64 residues (entire N-terminal domain) (*SI Appendix*, Fig. S5*B*).

Because deletion of the first 40 residues reduced binding and further deletion of residues 41 to 64 completely abolished binding, we created additional N-terminal deletions with starting points at amino acids 46, 48, 51, 56, and 60, which were cotransfected with PPM1G into cells. After Strep AP and Western blot analysis, we found that all these constructs completely abolished PPM1G binding (*SI Appendix*, Fig. S5*C*), suggesting that residues 41 to 45, at least, may participate in PPM1G binding. Given this knowledge, we then created internal ARF deletions to pinpoint short amino acid stretches that could contribute to PPM1G recognition. However, unexpectedly, we found that none of the internal ARF deletions alone interfered with PPM1G binding, indicating that even though residues 41 to 45 appear to be required for PPM1G association when the N terminal is lacking (*SI Appendix*, Fig. S5 *B* and *C*), they are apparently not necessary in the context of full-length ARF (*SI Appendix*, Fig. S5*D*).

Together, these data suggest that at least two distinct surfaces in ARF (short N terminal and internal) might contribute to PPM1G recognition (Fig. 6*A*). To test this possibility, we created double deletion constructs lacking β1 and α1 (Δβ1-α1, residues 1 to 11 and 41 to 55, respectively) or β2 and α1 (Δβ2-α1, residues 20 to 29 and 41 to 55, respectively). We then transfected these

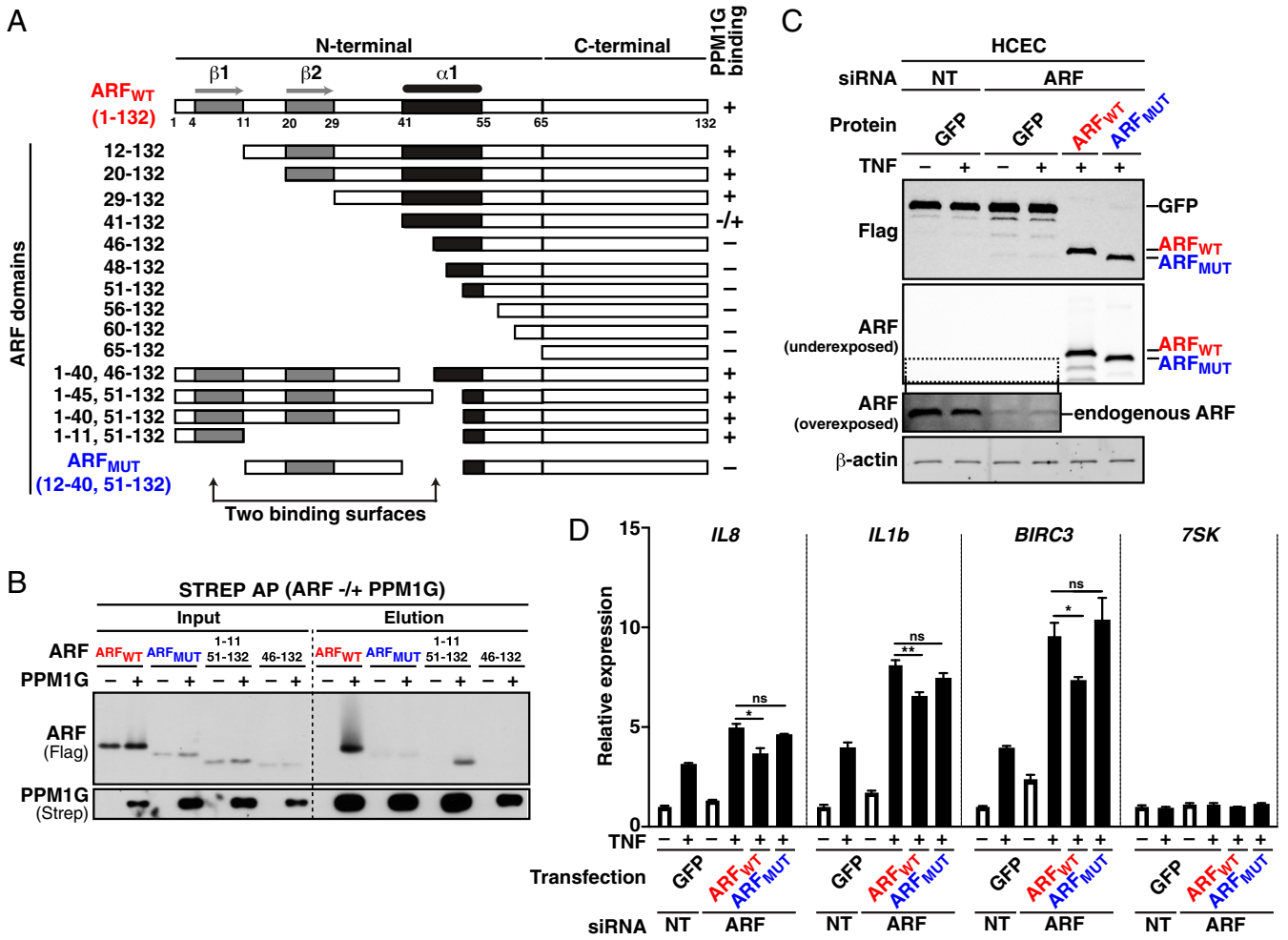

Fig. 6. ARF fine tunes TNF-induced, PPM1G-dependent NF-κB transcription. (*A*) Scheme of ARF secondary structure prediction with the protein domain arrangement including β1 sheet (residues 4 to 11), β2 sheet (residues 20 to 29), and α1 helix (residues 41 to 55). A summary of the PPM1G-binding properties of full-length ARF and domain deletions is shown to the right of the scheme (according to the results shown in *SI Appendix*, Fig. S5 and Fig. 6*B*). (*B*) Two ARF-binding surfaces mediate the ARF–PPM1G interaction. Strep-tagged PPM1G and Flag-tagged ARF (FL or deletion constructs) were cotransfected into HEK293T cells. Strep AP was performed followed by Western blot with the indicated antibodies to test whether the ARF deletion constructs had copurified with PPM1G. Data in all panels are representative of three independent experiments. (*C*) HCEC were transfected with siRNAs (NT or ARF), then retransfected with Flag-tagged GFP, wild-type ARF (ARF_{WT}), or the PPM1G-binding deficient mutant ARF (ARF_{MUT}), treated with or without TNF for 4 h, and then analyzed by Western blot with the indicated antibodies. (*D*) HCEC from panel *C* were used for RT-qPCR analysis of the indicated target genes and normalized to U6 (mean ± SEM are shown; $n = 4$). Statistical significance was calculated using unpaired Student's *t* test: $^{*}P < 0.05$, $^{**}P < 0.01$, ns, non-significant.

ARF mutants along with PPM1G, and after Strep AP, observed that while Δβ2-α1 still retained PPM1G-binding activity, the Δβ1-α1 construct (labeled as ARF_{MUT}) does not (Fig. 6 *A* and *B*). Interestingly, this collective evidence indicates that ARF uses a previously uncharacterized binding pattern composed of two binding interfaces (β1, residues 1 to 11 and α1, residues 41 to 45) to directly associate with PPM1G (Fig. 6*A*).

Given that 1) PPM1G is required for TNF-induced NF-κB transcription activation (16, 18), 2) ARF binds PPM1G (this study), and 3) ARF blunts NF-κB activity (ref. 28 and this study), we asked whether the ARF–PPM1G protein–protein interaction is required for the ARF-mediated phenotype. To test this, we first silenced ARF expression in HCEC with RNAi followed by ectopic expression of GFP (negative control), WT ARF (referred to as ARF_{WT}), or the PPM1G-binding deficient ARF mutant (ARF_{MUT}) (Fig. 6*C*) and then assessed the expression of NF-κB target genes upon TNF stimulation by RT-qPCR (Fig. 6*D*). Notably, unlike ARF_{WT}, ARF_{MUT} was unable to dampen TNF-induced NF-κB transcription activation of target genes (*IL8*, *IL1β*, and *BIRC3*) (Fig. 6*D*). Although we cannot precisely disregard that the nonfunctional ARF mutant has other interactions and functions interrupted, we provide evidence that reconstitution of ARF blunts NF-κB transcription activation in a PPM1G-dependent manner to normally control the magnitude of NF-κB target gene expression. Below we assess the clinical relevance of ARF-mediated regulation of NF-κB transcriptional programs in patient tumor data.

Loss of ARF in Tumors Is Associated with NF-κB–dependent, Chronic Inflammatory Signatures. Given the in vitro results, we predicted that loss of ARF in patient tumor samples would trigger an NF-κB–dependent inflammatory or antiapoptotic response. To test this idea, we data mined The Cancer Genome Atlas (TCGA) datasets to first identify solid tumors containing high prevalence of *ARF* alterations (both deletions and somatic mutations) for functional and pathway analysis. Because of the overlapping nature of the p14 and p16 ORFs in the *CDKN2A* locus (26), this analysis cannot precisely distinguish between alterations in p14 and/or p16, thus for simplicity we referred to *ARF* alterations despite this unambiguous classification. Among 32 solid tumors

showing *ARF* genetic alterations, we identified 14 tumors (GBM, HNSC, PAAD, ESCA, MESO, LUSC, BLCA, SKCM, DLBC, CHOL, LUAD, STAD, SARC, and LGG) with a high frequency of patients (>10%) bearing deletions and somatic mutations (Fig. 7*A*, see legend for tumor type abbreviation nomenclature).

Due to the low patient number with ARF deletions in CHOL ($n = 6$) and DLBC ($n = 13$), we excluded these tumor types from subsequent analysis in which we examined if loss of *ARF* is associated with deregulation of NF-κB gene expression programs (leaving 12 tumor types for downstream analysis). We also excluded tumors within these 12 tumor types containing somatic mutations since we were mainly interested in changes observed when *ARF* expression was lost and because mutations may give phenotypes unrelated to the mechanism studied herein. Of the 12 tumors, we first compared gene expression signatures in tumors containing wild-type or amplified *ARF* (WT + AMP) vs. those containing *ARF* deletions. Importantly, *ARF* WT + AMP tumors expressed much higher levels of ARF compared with tumors bearing *ARF* deletions (Fig. 7*B*), thus serving as an internal control that the tumor classification was accurate.

To interrogate whether *ARF* deletion in the 12 different tumor types having a high patient number with ARF deletions correlates with any transcriptional signatures, we compared the expression of all protein-coding genes in tumors between the two categories in each tumor type using a false discovery rate (FDR) <0.05. Expectedly, this analysis revealed a distinct number of down-regulated and up-regulated differentially expressed genes (DEGs) in the various tumor types (Dataset S2). Given the ARF-mediated dampening of TNF-induced gene expression, we were mainly interested in exploring the possibility of abnormal up-regulation of gene signatures in the *ARF* deletion tumor classification, which ranged between ∼100 and 6,000 genes depending on the tumor type (Dataset S2). Interestingly, Gene Ontology (GO) analysis discovered that 6 of the 12 tumor types interrogated (BLCA, HNSC, LGG, LUAD, PAAD, and SARC) have remarkable enrichment of terms related to inflammatory signatures including NF-κB signaling, response to TNF, and cellular response to IL-1β, among others (*SI Appendix*, Fig. S6).

To further test if there were any commonalities between the tumor types bearing the "chronic inflammatory signature," we performed an overlapping analysis for the genes up-regulated in the six tumor types bearing *ARF* deletions (BLCA, HNSC, LGG, LUAD, PAAD, and SARC). We then selected genes that were commonly enriched in at least three tumor types and subsequently performed GO analysis. Strikingly, this subset of commonly up-regulated genes (Dataset S3) were enriched with terms such as "positive regulation of NF-κB transcription factor activity" (q-value FDR Benjamini–Hochberg [BH] = 3×10^{-6}), "response to TNF" (q-value FDR BH = 7.39×10^{-5}), and "cellular response to IL-1β" (q-value FDR BH = 3.8×10^{-3}) (Fig. 7*C*). Other enriched pathways were "immune response" (q-value FDR BH = 1.09×10^{-4}), "regulation of cell death" (q-value FDR BH = 1.76×10^{-6}), and "WNT signaling pathway" (q-value FDR BH = 1.61×10^{-5}) (Fig. 7*C*). This analysis also identified a common set of unique genes commonly up-regulated and enriched in regulation of TNF superfamily cytokine production and immune responses, among others (Dataset S3), consistent with the in vitro data of NF-κB target gene deregulation upon ARF expression or loss (Figs. 3 and 5, respectively). Interestingly, these data argue that *ARF* deletion induces the activation of mutual genes among different types of tumors, suggesting that loss of ARF may be a biomarker for increased immunological responses in cancer. The idea that we observed some gene- and pathway-specific differences between the different tumor types tested is corroborated by the fact that we found differences in gene expression upon TNF stimulation in the presence/absence of ARF in the two cell culture systems interrogated (osteosarcoma and colon epithelial cells), potentially reflecting cell-type–specific gene programs.

Taken together, the integrated clinical analysis demonstrates that *ARF* loss in numerous types of cancer is associated with an NF-κB–dependent chronic inflammatory response and provides clinical relevance supporting the model that ARF is a negative regulator of PPM1G-dependent NF-κB transcription, by tuning the response to TNF.

Discussion

Precise NF-κB regulation is key for the control of multiple biological processes, including inflammatory responses, cell survival, and apoptosis. As such, regulation of this pathway is one mechanism by which organisms normally maintain tissue homeostasis to avoid malignancy (1). Like many enzymes that play key roles in the NF-κB transcriptional program including kinases (MSK1 and IKK) and histone acetyltransferases (p300 and CBP) (46, 47), PPM1G is a transcriptional coactivator of NF-κB in response to inflammatory and genotoxic insults (16, 18). PPM1G activates one of the major Pol II elongation kinases (CDK9) to promote transcription elongation of NF-κB target genes in response to inflammatory stimulation (48). Given this crucial role of PPM1G, we reasoned that its function must be tightly regulated to avoid excessive and/or extensive transcriptional outputs causing tissue damage (1, 2, 30).

In this work, we discovered that the *CDKN2A* tumor suppressor $p14^{ARF}$ assembles with PPM1G at a subset of NF-κB target gene promoters during inflammatory stimulation to restrain excessive activation of the NF-κB program. As such, we propose a model whereby ARF functions with PPM1G to provide "tunable control" of NF-κB transcription to regulate the inflammatory and apoptotic responses (*SI Appendix*, Fig. S7). In unstimulated cells, ARF constitutively binds PPM1G in the nucleus, but NF-κB target genes (proinflammatory and antiapoptotic) are not expressed or expressed at low levels because NF-κB is primarily inactivated in the cytoplasm (*SI Appendix*, Fig. S7*A*). Upon stimulation with TNF, NF-κB forms a ternary protein complex with ARF and PPM1G in the nucleus, leading to a basal level of expression of antiapoptotic and inflammatory genes that normally block TNF-induced apoptosis mediated by NF-κB–independent activation of caspases (*SI Appendix*, Fig. S7*A*). Cotreatment with TNF and agents that block NF-κB–dependent antiapoptotic factors (SMACm) enhances sensitivity to TNF-induced apoptosis (*SI Appendix*, Fig. S7*A*). In contrast, loss of ARF in TNF-stimulated conditions leads to excessive activation of antiapoptotic NF-κB target genes that bypass the SMACm-mediated inhibition, leading to partial resistance to TNF-induced apoptosis (*SI Appendix*, Fig. S7*B*). Interestingly, TCGA analysis showed that loss of ARF in cancer patients is indeed correlated with an enhanced inflammatory signature, suggesting that cancer cells may lose ARF to confer this antiapoptotic advantage and potentially provide resistance to TNF-induced apoptosis.

During the conception of our studies, we envisioned two potential models of ARF-mediated regulation. In model 1, ARF could be a "kinetic regulator," assembling into the ternary complex and being recruited to NF-κB target genes in response to TNF simulation in the later stages of NF-κB activation (deactivation stage). In model 2, ARF could be a "tuner regulator" of NF-κB target gene expression, whereby ARF is recruited to NF-κB target genes in response to TNF stimulation as part of the ternary complex in the early stages of NF-κB activation. Given the fact that ARF and PPM1G interact constitutively in the nucleus (Fig. 4) and that ARF ectopic expression (and loss) leads to deregulation of the NF-κB–dependent transcription at both early and late stages (Figs. 3 and 5), we propose that ARF provides tunable control of TNF-induced stimulation and cell fate responses.

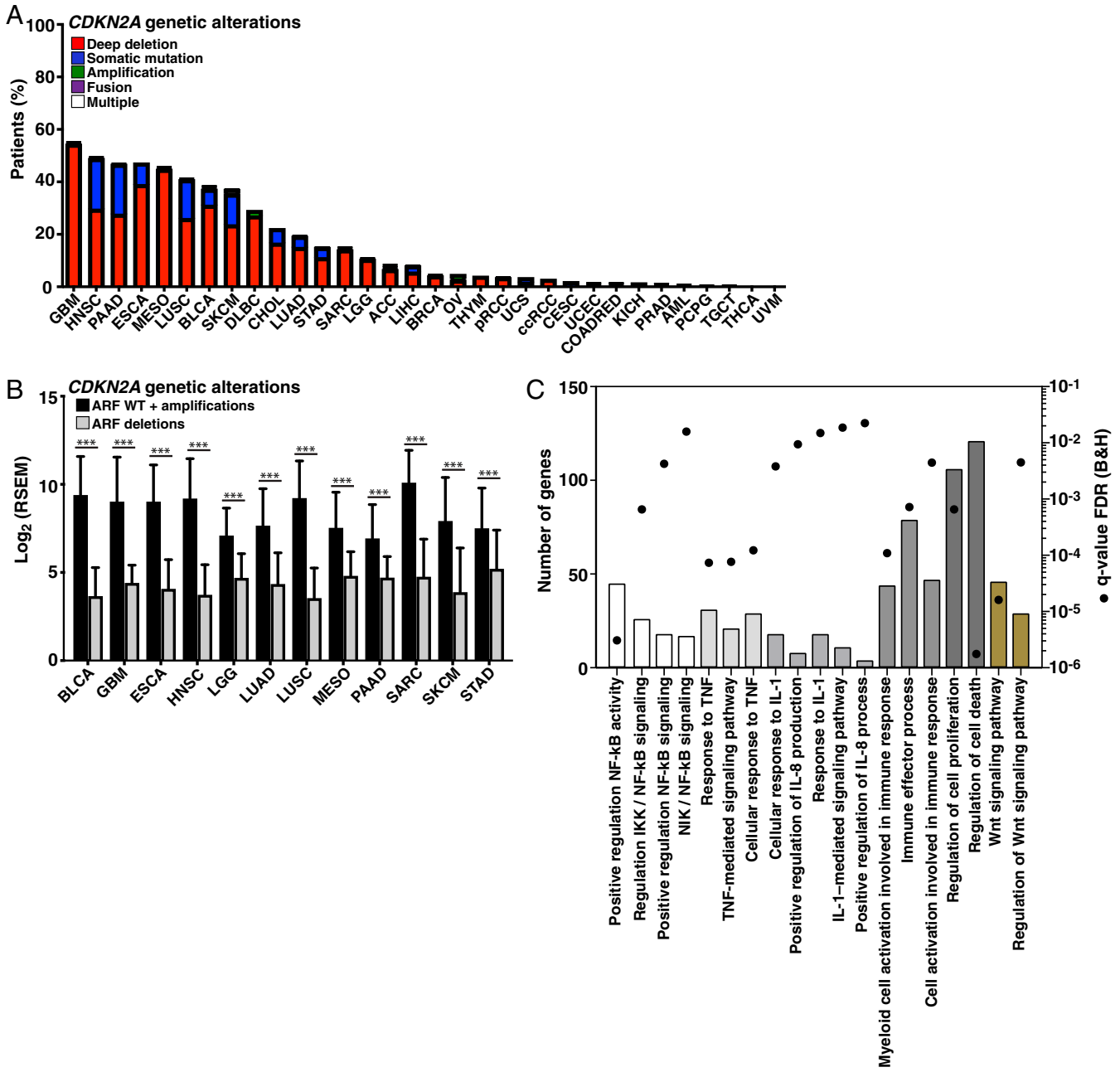

Fig. 7. Tumors of patients with ARF deletions reveal NF-κB–dependent chronic inflammatory signatures. (*A*) Frequency of *ARF* alterations per tumor type as obtained from TCGA bioportal. (*B*) *ARF* gene expression (Log_2 RNA-seq by expectation maximization [RSEM]) in WT plus ARF amplifications vs. *ARF*-deleted tumors per tumor type. Statistical significance was calculated using unpaired Student's *t* test: $^{***}P < 0.001$. (*C*) Enrichment of gene signatures associated with inflammatory and immune responses in commonly up-regulated genes in ARF-deleted vs. WT plus ARF amplified tumors. Commonly up-regulated genes were defined as being up-regulated in at least three of the following tumors: BLCA, HNSC, LGG, LUAD, PAAD, and SARC. The number of genes in each defined pathway is indicated by the bar graph. The q-value defining the significance of each term is reported as a dot on each term. Abbreviations: acute myeloid leukemia (AML), adrenocortical carcinoma (ACC), bladder urothelial carcinoma (BLCA), brain lower grade glioma (LGG), breast invasive carcinoma (BRCA), cervical and endocervical cancers (CESC), cholangiocarcinoma (CHOL), colorectal adenocarcinoma (COADREAD), esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (ccRCC), kidney renal papillary cell carcinoma (pRCC), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), lymphoid neoplasm diffuse large B cell lymphoma (DLBC), mesothelioma (MESO), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), pheochromocytoma and paraganglioma (PCPG), prostate adenocarcinoma (PRAD), sarcoma (SARC), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), testicular germ cell tumors (TGCT), thymoma (THYM), thyroid carcinoma (THCA), uterine carcinosarcoma (UCS), uterine corpus endometrial carcinoma (UCEC), and uveal melanoma (UVM).

Importantly, our data provide a mechanistic explanation for how ARF controls inflammation and cell death by impinging on the transcription elongation machinery thereby interfering with its function (17). Interestingly, we also found that PPM1G directly binds and promotes ARF stabilization. While we propose that this stabilization may facilitate ARF functions in the regulation of NF-κB–dependent transcription, more work is needed to define the mechanistic basis. In addition, ARF

signaling is complex and involves p53-dependent and -independent pathways mainly aiming at restraining abnormal cell growth and at maintaining genomic stability (49). Thus, by no means are we ruling out that part of the observed transcriptional changes and functional consequences are contributed by these other well-known functions.

Since extracellular stimuli signal through the cytoplasm to chromatin through histone modifications (50), it is likely that ARF functions in the NF-κB circuit are regulated by both cytoplasmic and nuclear events. In fact, in transformed cell lines, part of ARF-mediated repression of NF-κB function has been shown to require the ATR kinase (28, 29). In addition to this signaling layer of control, our biochemical and genetic data reveal a nuclear, PPM1G-dependent, ARF-mediated regulatory event in restraining the NF-κB transcriptional program. Since PPM1G is a phosphatase, one obvious hypothesis that we initially tested was whether ARF binds PPM1G to block its catalytic function (particularly with its phosphatase activity on the P-TEFb kinase). However, after several biochemical attempts and reconstitution of stoichiometric PPM1G–ARF protein complexes, we did not find any evidence that ARF would block the phosphatase function in vitro. Additionally, ARF loss did not affect PPM1G interactions with other known cofactors such as HEXIM1, Larp7, CDK9, and endogenous NF-κB (*SI Appendix*, Fig. S4*F*), indicating that ARF does not regulate PPM1G interaction with NF-κB and these critical cofactors. Given these results, it is possible that ARF controls PPM1G coactivator function at other levels. For example, ARF may directly bind chromatin at selected loci to prevent PPM1G-dependent aberrant, chronic inflammatory signaling. Additionally, given that PPM1G binds the H2A/H2B dimer (27) and that ARF selectively binds H2B tails to induce chromatin silencing (51), ARF could redirect PPM1G, and potentially other unknown cofactors, to combinatorially bind histone tails to "tune" the expression of genes within the inflammatory program.

In support of our findings that ARF acts as a "tuner" in the inflammatory response, ARF has been reported to play vital roles in the regulation of many biological processes, including antiviral, innate immune, and inflammatory cell signaling responses (52–55). ARF is induced by IFN and viral infections, and *ARF*-deficient mice are hypersensitive to viral infections, thereby suggesting a protective role of ARF in this context (52). Consistent with its antiinflammatory function, the induction of antiinflammatory mediators in peritoneal and bone marrow-derived macrophages from *ARF*-deficient animals in response to Toll-like receptor ligands was severely impaired (53). In addition, *ARF*-deficient mice showed an enhancement in the level of tumor-associated macrophages with antiinflammatory and immune-suppressive properties, which are known to promote tumor proliferation and cancer cell spreading (54–56), thereby potentially opening the possibility of a broad ARF-mediated NF-κB regulatory function in various cell types and tissues. Despite these physiologically relevant descriptions, the mechanism by which ARF functions to control innate immunity and inflammatory responses is incompletely understood. Even though ARF is induced by IFN and viral infections (52–55), we found no evidence (in several cancer cell lines, HCEC, primary human monocytes, and $CD4^+$ T cells) of TNF-mediated *ARF* induction at the transcriptional and posttranscriptional levels.

In summary, although our discoveries provide mechanistic insight into the tumor suppressive and antiinflammatory functions of ARF, important questions remain for our full understanding of ARF functions in the context of normal and disease states. For example, given that our studies were performed in a cancer cell model in which ARF was ectopically expressed (NARF2-E6) and normal cells in which endogenous ARF was silenced (HCEC), future studies will assess whether ARF loss in a broad panel of cancer cells leads to similar phenotypic and molecular readouts. In addition, given that ARF is expressed in many tissues, an interesting scenario would be to interrogate possible tissue-specific ARF functions in the absence and presence of inflammatory conditions. It would also be interesting to test whether simultaneous loss of *ARF* and appearance of inactivating genetic alterations in NF-κB signaling components (e.g., gain of function in activating subunits and/or loss of function in inhibitory subunits) lead to oncogenic activation of NF-κB (57). Additionally, given that the ARF–PPM1G–NF-κB ternary complex appears to function on chromatin, future research will help clarify what the underlying molecular bases are for target gene specificity. As shown here, since ARF expression, subcellular localization, and function are compromised in cancer cell lines, normal cells that are genetically tractable (like HCEC) provide physiological systems to answer these outstanding questions. By compiling this information, we will start elucidating the basis of noncanonical mechanisms by which the ARF tumor suppressor functions in the control of immunity, inflammation, and apoptosis, all of which are particularly important to restraining and regulating the tumor landscape at multiple levels. It is well appreciated that other tumor suppressors play multiple roles in the control of normal cellular function that go beyond restraining cell growth to block tumorigenesis such as the direct control of metabolic and immune processes (58). As such, these studies open avenues of investigation with significant relevance for biomedical research.

Materials and Methods

A detailed description of cloning and plasmid generation, DNA and RNA transfections, RT-PCR, AP and IP assays, AP-MS analysis, immunofluorescence, ChIP-qPCR, FACS, and TCGA analysis are provided in *SI Appendix, Materials and Methods*. Tables are also provided in *SI Appendix* for cell stocks (*SI Appendix*, Table S1), plasmid generations (*SI Appendix*, Table S2), DNA oligonucleotides (*SI Appendix*, Table S3), and antibodies (*SI Appendix*, Table S4). The following datasets can also be downloaded for mass spectrometry results (Dataset S1), lists of DEGs between tumors bearing ARF WT + AMP or deleted ARF (Dataset S2), and lists of a core set of frequently up-regulated NF-κB target genes in various tumor types upon loss of *ARF* expression (Dataset S3).

Cell Culture. HEK293T, HeLa, and NARF2-E6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C with 5% CO_2. HCEC 1CT cells were cultured according to ref. 37.

Statistical Analysis. Error bars represent ± SEM; *P* values were calculated with GraphPad Prism V7.04 using the statistical test described for each experiment unless otherwise indicated.

TCGA Analysis. Clinical information, gene expression, mutations, and copy number analysis (RNA sequencing [RNA-seq]) data were obtained from TCGA for 32 cancer types using the FIREBROWSE portal (http://firebrowse.org/).

Data Availability. All study data are included in the article and supporting information.

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