

TRIM28 Is an E3 Ligase for ARF-Mediated NPM1/B23 SUMOylation That Represses Centrosome Amplification

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The tumor suppressor ARF enhances the SUMOylation of target proteins; however, the physiological function of ARF-mediated SUMOylation has been unclear due to the lack of a known, associated E3 SUMO ligase. Here we uncover TRIM28/KAP1 as a novel ARF-binding protein and SUMO E3 ligase for NPM1/B23. ARF and TRIM28 cooperate to SUMOylate NPM1, a nucleolar protein that regulates centrosome duplication and genomic stability. ARF-mediated SUMOylation of NPM1 was attenuated by TRIM28 depletion and enhanced by TRIM28 overexpression. Coexpression of ARF and TRIM28 promoted NPM1 centrosomal localization by enhancing its SUMOylation and suppressed centrosome amplification; these functions required the E3 ligase activity of TRIM28. Conversely, depletion of ARF or TRIM28 increased centrosome amplification. ARF also counteracted oncogenic Ras-induced centrosome amplification. Centrosome amplification is often induced by oncogenic insults, leading to genomic instability. However, the mechanisms employed by tumor suppressors to protect the genome are poorly understood. Our findings suggest a novel role for ARF in maintaining genome integrity by facilitating TRIM28-mediated SUMOylation of NPM1, thus preventing centrosome amplification.

The *INK4a/ARF* locus encodes two tumor suppressors, (i) p16^{INK4a}, an inhibitor of cyclin-dependent kinase 4/6 (CDK4/6), and (ii) an alternative reading frame (ARF) protein (p14^{ARF} in humans and p19^{ARF} in mouse), referred to here as “ARF.” This locus is mutated in approximately 40% of human cancers. ARF contributes to p53 stabilization and activation through inhibition of murine double minute 2 (MDM2), leading to cell cycle arrest, apoptosis, or senescence. Oncogenic insults such as mutations in Ras upregulate the expression of ARF to protect cells from tumorigenesis (1).

There is substantial evidence that ARF has additional, p53-independent functions (2). For example, a broader spectrum of tumors was observed in *ARF/p53*-null mice than in *p53*-null mice (3). ARF is enriched within the nucleolus but also localizes to the mitochondria to induce apoptosis or autophagy in a p53-independent manner (2). These data support a role of ARF in p53-independent tumor-suppressive functions. However, the underlying mechanisms remain elusive.

Interestingly, ARF has been shown to promote SUMOylation of MDM2 (4), nucleophosmin (NPM1/B23) (5), and the Werner helicase (WRN) (6) independently of p53. However, ARF did not stimulate SUMOylation in an *in vitro* reconstitution reaction in the presence of SUMO E1/E2 enzymes (6), suggesting that ARF is not the E3 SUMO ligase. The mechanism and biological impact of ARF-mediated SUMOylation are currently unclear.

NPM1 has been shown to be involved in the p53-independent ARF pathway (7). NPM1 usually resides in the nucleolus but also shuttles between the nucleus and cytoplasm (8). SUMOylation of NPM1 contributes to its centrosomal localization (9), and NPM1 has been reported to exert tumor-suppressive function. For example, loss of NPM1 leads to centrosome amplification, resulting in genome instability (10).

The tripartite motif-containing (TRIM) protein family contains a RING domain at the N terminus and has diverse biological roles. TRIM28, also known as Krüppel-associated box (KRAB)-associated protein 1 (KAP1) and transcription intermediary factor

1 β (TIF1 β), has been shown to act as an E3 SUMO ligase for itself (11), IRF7 (12), and Vps34 (13).

In this study, we have identified TRIM28 as a novel binding partner of ARF. We found that TRIM28 is an E3 SUMO ligase responsible for ARF-mediated SUMOylation of NPM1. Increased NPM1 SUMOylation by ARF/TRIM28 contributes to its enhanced centrosomal localization and suppression of centrosome amplification, suggesting a novel, p53-independent tumor-suppressive function of ARF.

MATERIALS AND METHODS

Plasmids, adenoviruses, and retroviruses. Full-length TRIM28 cDNA was purchased from Open Biosystems (GenBank accession number BC004978; Waltham, MA), and untagged full-length TRIM28 and FLAG-tagged full-length TRIM28 (amino acids 1 to 832) were cloned into vector pcDNA3.1⁽⁺⁾ (Invitrogen). All cloned constructs were confirmed by direct DNA sequencing. ARF constructs, NPM1 constructs, MDM2 constructs, adenovirus infection, and retrovirus infection have been described elsewhere (7, 14, 15). Recombinant adenoviruses encoding untagged TRIM28, untagged ARF, FLAG-tagged ARF, FLAG-tagged ARF (amino acids 14 to 132), and green fluorescent protein (GFP) were produced as previously described (7) using the AdEasy XL adenoviral vector

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system (Agilent Technologies). Deletion constructs and point mutations in TRIM28 and NPM1 were introduced by PCR-based site-directed mutagenesis. TRIM28 was also cloned into the pET3E-His vector. Full-length ARF was cloned into the pGEX vector (Amersham Bioscience) to generate the expression plasmid for glutathione *S*-transferase (GST)-ARF. Full-length SUMO3 was cloned into pcDNA3.1⁽⁺⁾ to express His₆-SUMO3. The pRetroSuper (pRS)-shARF construct was provided by Mathijs Voorhoeve (Duke-NUS, Singapore) for the generation of retrovirus, as previously described (16). The pGEX3X-NPM1 construct was provided by Mikael Lindström (Karolinska Institutet, Sweden). Transfection was carried out using ScreenFect A (Wako) or FugeneHD (Promega) according to the manufacturer's instructions.

Cell culture. U2OS and Saos-2 osteosarcoma, H1299 lung carcinoma, and NIH 3T3 fibroblast cell lines (ATCC) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Wild-type, *p53*^{-/-}, *p53*^{-/-}; *Mdm2*^{-/-}, *ARF*^{-/-}, and *p53*^{-/-}; *ARF*^{-/-} mouse embryonic fibroblasts (MEFs) kindly provided by Yanping Zhang (University of North Carolina, Chapel Hill, NC) were cultured similarly and used between passages 2 and 5.

RNA interference. Cells were transfected with short interfering RNAs (siRNAs) targeting TRIM28 or Silencer negative control #1 siRNA (Invitrogen) using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. The sequence of siRNA used to target the TRIM28 gene, si-TRIM28, is 5'-GCGGAAAUGUGAGCGUGUAdtdt-3'.

Protein analysis. Antibodies against FLAG (M2; Sigma), TRIM28 (20C1; Abcam), 6×His (MBL), Myc (ab9106 from Abcam and 9E10 from Santa Cruz Biotechnology), hemagglutinin (HA) (TANA2; MBL and F7; Santa Cruz Biotechnology), GST (B-14; Santa Cruz Biotechnology), NPM1 (FC-61991; Invitrogen), p19ARF (5-C3-1; Santa Cruz Biotechnology), tubulin (DM1A+DM1B; Abcam), Ras (3965; Cell Signaling), histone H3 (9715; Cell Signaling), p53 (DO1; Santa Cruz Biotechnology), MDM2 (4B11; Santa Cruz Biotechnology), and β-actin (C4; Millipore) were purchased commercially. Rabbit polyclonal anti-p14ARF antibody was produced using C-terminal ARF sequence as the epitope (17). Cells were lysed in 0.1% NP-40 lysis buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], and protease inhibitor cocktail) for immunoprecipitation and in 2% SDS lysis buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol, and 2% SDS) for Western blotting as previously described (7). Chemiluminescent signal from horseradish peroxidase (HRP)-labeled secondary antibodies was detected using detection reagents according to the manufacturer's instructions (Pierce). Signal from fluorescence-labeled secondary antibodies was detected using the Odyssey Infrared Imaging system (LI-COR Biosciences, USA).

Immunoprecipitation. For liquid chromatography/mass spectrometry analysis (LC-MS), cells were lysed in 0.1% NP-40 lysis buffer and immunoprecipitated with anti-FLAG antibody conjugated to agarose beads (Sigma). Protein complexes were eluted with 3×FLAG peptide (Sigma). The affinity-purified protein complexes were subjected to insoluble trypsin digestion for LC-MS. For other immunoprecipitations, antibodies were added to precleared lysate and incubated overnight at 4°C. Subsequently, 20 μl protein A/G-agarose beads (Pierce) was added to the reaction mixture, which was incubated for 2 h at 4°C. The beads were washed three times with 0.1% NP-40 lysis buffer. Protein complexes were eluted with 2% SDS lysis buffer, resuspended in loading buffer, and analyzed by Western blotting.

Mass spectrometry and data analysis. Eluted protein complexes were digested in solution with trypsin, applied to a C₁₈ column (Pierce), washed with ultrapure water to remove any salt, eluted with acetonitrile, and concentrated using a SpeedVac vacuum concentrator. Samples were analyzed by LC-tandem mass spectrometry (LC-MS/MS). MS data were analyzed using GPM software (http://p3.thegpm.org/tandem/thegpm_ppp.html) and default parameter settings.

Expression and purification of recombinant proteins. Expression of GST-ARF, GST-NPM1, and His₆-TRIM28 was induced in *Escherichia coli*

BL21(DE3) cells for 4 h at 37°C using 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Bacterial cells expressing GST fusion proteins were lysed in sonication buffer (50 mM Tris HCl [pH 8.0], 0.5 M NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 1 mM dithiothreitol [DTT], protease inhibitors, and 1 mg/ml lysozyme) and sonicated on ice for 20 s six times. The lysate was centrifuged at 12,000 × g for 10 min. GST fusion proteins were then purified using glutathione-Sepharose beads (Pierce) and used in the bead-conjugated form or eluted in 10 mM reduced glutathione and dialyzed against phosphate-buffered saline (PBS). Bacterial cells expressing His₆-TRIM28 were lysed in sonication buffer and sonicated on ice for 20 s, six times. His₆-TRIM28 was bound to Ni²⁺-nitrilotriacetic acid (Ni²⁺-NTA) beads (Pierce), eluted in 500 mM imidazole, and dialyzed against phosphate-buffered saline (PBS).

In vitro GST assay. GST-tagged fusion proteins loaded onto glutathione-Sepharose beads were incubated with purified His₆-tagged protein overnight at 4°C. Beads were collected by centrifugation and washed extensively with 50 mM HEPES (pH 7.4) containing 200 mM NaCl, 5 mM MgCl₂, 0.5% Tween 20, 1 mM DTT, and protease inhibitors, and bound proteins were separated by SDS-PAGE for Western blotting.

In vivo SUMOylation assay. Plasmids encoding His₆-SUMO3, TRIM28, ARF, HA-NPM1, and Myc-NPM1 were cotransfected into cells. At 48 h posttransfection, cells from each plate were harvested and placed in two aliquots. One aliquot (approximately 20% of cells) was lysed in 2% SDS lysis buffer for Western blotting to confirm expression of transfected proteins. The remaining cells were lysed in denaturing lysis buffer (6 M guanidinium-HCl, 0.1 M Na₂HPO₄-NaH₂PO₄ [pH 8.0], 0.01 M Tris-HCl [pH 8.0], 5 mM imidazole, and 10 mM β-mercaptoethanol) and incubated with Ni²⁺-NTA beads (Pierce) for 4 h at room temperature. Beads were sequentially washed with buffer A (6 M guanidinium-HCl, 0.1 M Na₂HPO₄-NaH₂PO₄ [pH 8.0], 0.01 M Tris-HCl [pH 8.0], and 10 mM β-mercaptoethanol), buffer B (8 M urea, 0.1 M Na₂HPO₄-NaH₂PO₄ [pH 8.0], 0.01 M Tris-HCl [pH 8.0], and 10 mM β-mercaptoethanol), and buffer C (8 M urea, 0.1 M Na₂HPO₄-NaH₂PO₄ [pH 6.3], 0.01 M Tris-HCl [pH 6.8], 10 mM β-mercaptoethanol, and 0.2% Triton X-100) followed by elution of bound proteins with buffer D (200 mM imidazole, 0.15 M Tris-HCl [pH 6.8], 30% glycerol, 0.72 M β-mercaptoethanol, and 5% SDS). Eluted proteins were suspended in loading buffer and separated by SDS-PAGE for Western blotting.

In vitro SUMOylation assay. The SUMOylation assay was carried out by using the SUMOylation assay kit according to the manufacturer's instructions (Abcam). GST-ARF, GST-NPM1, and His₆-TRIM28 recombinant proteins from bacteria were incubated with SUMO3, SUMO E1 activating enzyme, and SUMO E2 conjugating enzyme at 30°C for 2 h. The reaction was stopped by adding SDS-PAGE loading buffer, and the results were analyzed by Western blotting.

Immunofluorescence. For staining centrosome with rabbit anti-γ-tubulin (Abcam), cultured cells were fixed in 10% neutral buffered formalin (Sigma) for 10 min, washed twice with PBS, permeabilized in cold PBS containing 0.2% Triton X-100 for 5 min, and incubated with blocking buffer (PBS containing 0.5% bovine serum albumin [BSA]) for 30 min prior to incubation with anti-γ-tubulin in blocking buffer for 1 h. Cells were then incubated with DyLight Fluor 488-conjugated secondary antibody (Jackson ImmunoResearch) for 30 min, washed three times with PBS, and counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Stained cells were examined with an Olympus model IX71 inverted microscope fitted with appropriate fluorescence filters.

To determine centrosome number, centrosome NPM1, and transfected cells, cells were fixed in 10% formalin for 10 min and then in methanol for 10 min, washed in PBS, and blocked for 1 h in blocking buffer. Cells were coimmunostained overnight with goat anti-γ-tubulin (Abcam), rabbit anti-NPM1 (PabN1) (gift of Kenji Fukasawa, H. Lee Moffitt Cancer Center, Tampa, FL) (18, 19), and mouse anti-FLAG (Sigma) against FLAG-TRIM28 or mouse anti-Myc (Santa Cruz Biotechnology) against Myc-ARF. They were subsequently incubated with DyLight 488-, Rhodamine Red 594-, and Dylight 405-conjugated secondary

antibodies (Jackson ImmunoResearch), respectively, for 30 min. Stained cells were washed with PBS and examined with a Carl Zeiss LSM 710 upright confocal microscope.

Duolink *in situ* PLA. *In situ* proximity ligation assay (PLA) was performed to detect protein-protein interactions using the Duolink II detection kit according to the manufacturer's protocol (Olink Bioscience). Briefly, fixed cells were incubated with the primary antibodies mouse anti-FLAG (M2) and rabbit anti-ARF. Oligonucleotide-conjugated secondary antibodies against each of the primary antibodies were added. The oligonucleotides, which generate a green signal where ARF and TRIM28 are in close proximity (<40 nm apart), were ligated and amplified. Samples were examined using an Olympus IX71 inverted microscope fitted with appropriate fluorescence filters.

Metaphase spread analysis. Cells were cultured on the cover glass and incubated with 100 ng/ml of colcemid for 5 h (wild-type MEFs) or 10 ng/ml of colcemid overnight (*ARF*^{-/-}, *p53*^{-/-}, and *p53*^{-/-}; *ARF*^{-/-} MEFs) together with 100 μ M BrdU. Cells were then treated with 0.8% sodium citrate for 1 h at 37°C. After fixation in three changes of methanol-acetic acid (3:1), chromosome spreads were made by firmly tapping with moderate force and air drying. Images of chromosome spread (z-sections of 250-nm intervals) were acquired using a confocal microscopy system (Carl Zeiss LSM 710; Jena, Germany) equipped with a 405-nm laser, an oil-immersion objective lens (numerical aperture [NA], 1.40, 63 \times ; Plan Achromat, Carl Zeiss), and ZEN 2010 software (version 6.0.0.485; Carl Zeiss). The maximum intensity projection along the z axis or three-dimensional (3D) projection of the images was generated using ImageJ software (version 1.45f). Chromosome counts were obtained from three independent experiments, and 20 metaphase chromosome spreads were counted in each experiment using 3D projection of the images. Representative images of the chromosome spread were presented as maximum intensity projection along the z axis.

Statistical analysis. The results were expressed as means \pm standard deviations (SD). Two-tailed unpaired Student's *t* test was used for comparing test data values with control values.

RESULTS

TRIM28 is a novel binding partner of ARF. To uncover p53-independent functions of ARF, we first identified its binding partners. We generated an adenovirus (Ad) to overexpress FLAG-tagged human ARF (amino acids 14 to 132) that lacks an MDM2-binding site and thus lacks the ability to induce p53-dependent cell cycle arrest (20). We performed a large-scale coimmunoprecipitation (co-IP) following overexpression of human ARF(14-132)-FLAG in Saos-2 osteosarcoma cells (*p53*-null). The ARF (14-132)-FLAG immunocomplexes were analyzed by liquid chromatography-mass spectrometry, and several polypeptides that specifically copurified with ARF(14-132)-FLAG but not with control GFP were identified (see Fig. S1 in the supplemental material). Among these peptides, we were particularly interested in a known E3 SUMOylation ligase, nuclear protein TRIM28, since the mechanism for ARF-mediated SUMOylation of target proteins was unknown.

To validate the *in vivo* interaction of ARF and TRIM28, co-IP of endogenous TRIM28 and ectopically expressed ARF was performed using lysates of U2OS cells (which do not express endogenous ARF) (Fig. 1A). Binding was also confirmed reciprocally by co-IP of ectopically expressed FLAG-TRIM28 and ARF in U2OS cells (Fig. 1B). The ARF-TRIM28 interaction was further demonstrated under physiological conditions. Endogenous TRIM28 coimmunoprecipitated with endogenous ARF in H1299 cells expressing control GFP short hairpin RNA (shRNA) (*p53*-null) (Fig. 1C). The amount of coimmunoprecipitated TRIM28 by ARF was reduced in H1299 cells expressing ARF shRNA (16), correspond-

ing to the reduced amount of ARF pulled down in the immunocomplex (Fig. 1C). Since MDM2 has been shown to associate with TRIM28 (21) and also with ARF, we next determined if the ARF-TRIM28 interaction was direct and independent of MDM2. GST-ARF associated with His-TRIM28 in a pulldown assay, demonstrating a direct interaction (Fig. 1D). In addition, ectopically expressed ARF coimmunoprecipitated with endogenous TRIM28 from *p53*^{-/-}; *Mdm2*^{-/-} MEFs (Fig. 1E). These results suggest that the ARF-TRIM28 interaction is independent of p53 or MDM2. The ARF-TRIM28 interaction was also observed using wild-type MEF (see Fig. S2 in the supplemental material), indicating that the presence of p53 and Mdm2 does not preclude the ARF-TRIM28 association. Lastly, we performed a proximity ligation assay, which confirmed that TRIM28 and ARF interact and their interaction is in the nucleus (Fig. 1F).

The ARF N' terminus binds the coiled-coil region of TRIM28. To determine the domains involved in the ARF-TRIM28 interaction, we constructed a series of Myc-ARF deletion mutants, overexpressed them in U2OS cells (ARF negative), and evaluated binding to endogenous TRIM28 by co-IP. We found that the region of ARF responsible for binding to TRIM28 is the exon 1 β -encoded N-terminal domain (amino acids 18 to 64) (Fig. 2A). Similarly, FLAG-TRIM28 deletion mutants were cotransfected with Myc-ARF into U2OS cells. We found that the coiled-coil domain of FLAG-TRIM28 (amino acids 239 to 381) was sufficient to immunoprecipitate with Myc-ARF (Fig. 2B). The coiled-coil region is also involved in MDM2 binding (21). In summary, these results suggest that the ARF N-terminal region (amino acids 18 to 64) binds to the TRIM28 coiled-coil domain (amino acids 239 to 381).

TRIM28 is an E3 ligase for ARF-mediated SUMOylation of NPM1. ARF has been shown to bind NPM1, thus enhancing its SUMOylation *in vivo* (5); however, it is unable to catalyze SUMOylation *in vitro* (6). As TRIM28 has been identified as an E3 SUMOylation ligase, we hypothesized that TRIM28 SUMOylates NPM1 by cooperating with ARF. To this end, we performed an *in vitro* SUMOylation assay. In the presence of SUMO3, SUMO E1 activating enzyme, and SUMO E2 conjugating enzyme, ARF itself was not able to catalyze the SUMOylation of NPM1, as reported (6), confirming that ARF does not possess E3 SUMO ligase activity (Fig. 3A, lane 2). On the other hand, NPM1 SUMOylation was observed when TRIM28 was added to the reaction mixture with these components (lane 3), and the SUMOylation was further enhanced by the addition of both TRIM28 and ARF (lane 4). SUMOylation required ATP and magnesium (lane 5). These data strongly suggest that TRIM28 is a SUMO E3 ligase for NPM1 and that ARF enhances TRIM28-mediated SUMOylation.

To verify that endogenous ARF is required for NPM1 SUMOylation *in vivo*, we engineered H1299 cells that ectopically express Myc-NPM1, His-SUMO3, and shARF to knock down ARF expression or shGFP as a control and performed an *in vivo* SUMOylation assay. We observed a decrease in SUMOylation of Myc-NPM1 when endogenous ARF expression was reduced by shARF (Fig. 3B, compare lanes 3 and 4). The contribution of endogenous ARF to NPM1 SUMOylation was further confirmed in mouse embryonic fibroblasts (MEFs) (Fig. 3C) and Saos-2 cells (Fig. 3D). Conversely, overexpression of ARF induced NPM1 SUMOylation (Fig. 3E, lane 1 and 3), and overexpression of both TRIM28 and ARF dramatically increased SUMOylation of NPM1 in H1299 cells (Fig. 3E, lane 4), suggesting that ARF and TRIM28 cooperate in NPM1 SUMOylation. In addition, siRNA-mediated

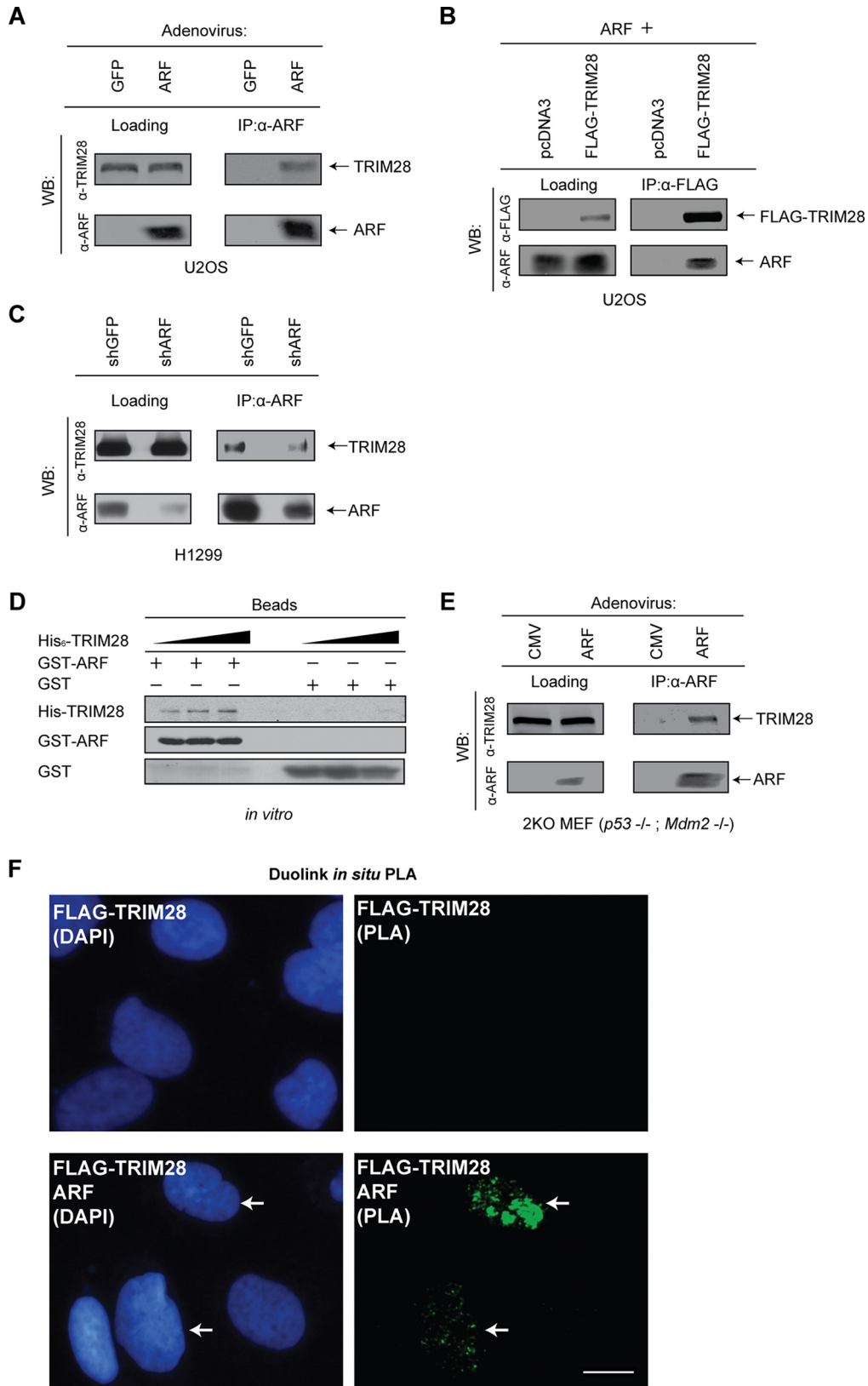


FIG 1 TRIM28 is a novel binding partner of ARF. (A and B) Ectopically expressed ARF binds TRIM28. U2OS cells were infected with adenoviruses encoding the indicated proteins (A) or transfected with the indicated plasmids (B). Immunoprecipitation (IP) was performed with the indicated antibodies and analyzed by Western blotting (WB). (C) ARF binds endogenous TRIM28. Lysates of H1299 cells expressing retrovirus-encoded control GFP short hairpin RNA (shRNA) or ARF shRNA were immunoprecipitated with anti-ARF antibody. (D) Direct interaction between ARF and TRIM28. Purified bacterial recombinant His₆-TRIM28 was mixed with GST-ARF, followed by pulldown with glutathione beads. The resulting complexes were analyzed by Western blotting. (E) ARF binds TRIM28 independently of both p53 and MDM2. Cell lysates from Ad-ARF-infected *p53*^{-/-}; *Mdm2*^{-/-} MEFs were immunoprecipitated with anti-ARF antibody. (F) ARF binds TRIM28 in the nucleus. U2OS cells were transfected with the indicated plasmids and analyzed by Duolink PLA. Cells with positive PLA signals (green [indicated by arrows], right panels) indicates ARF-TRIM28 binding. Left panels show DAPI-stained nuclei. Scale bar, 3 μm.

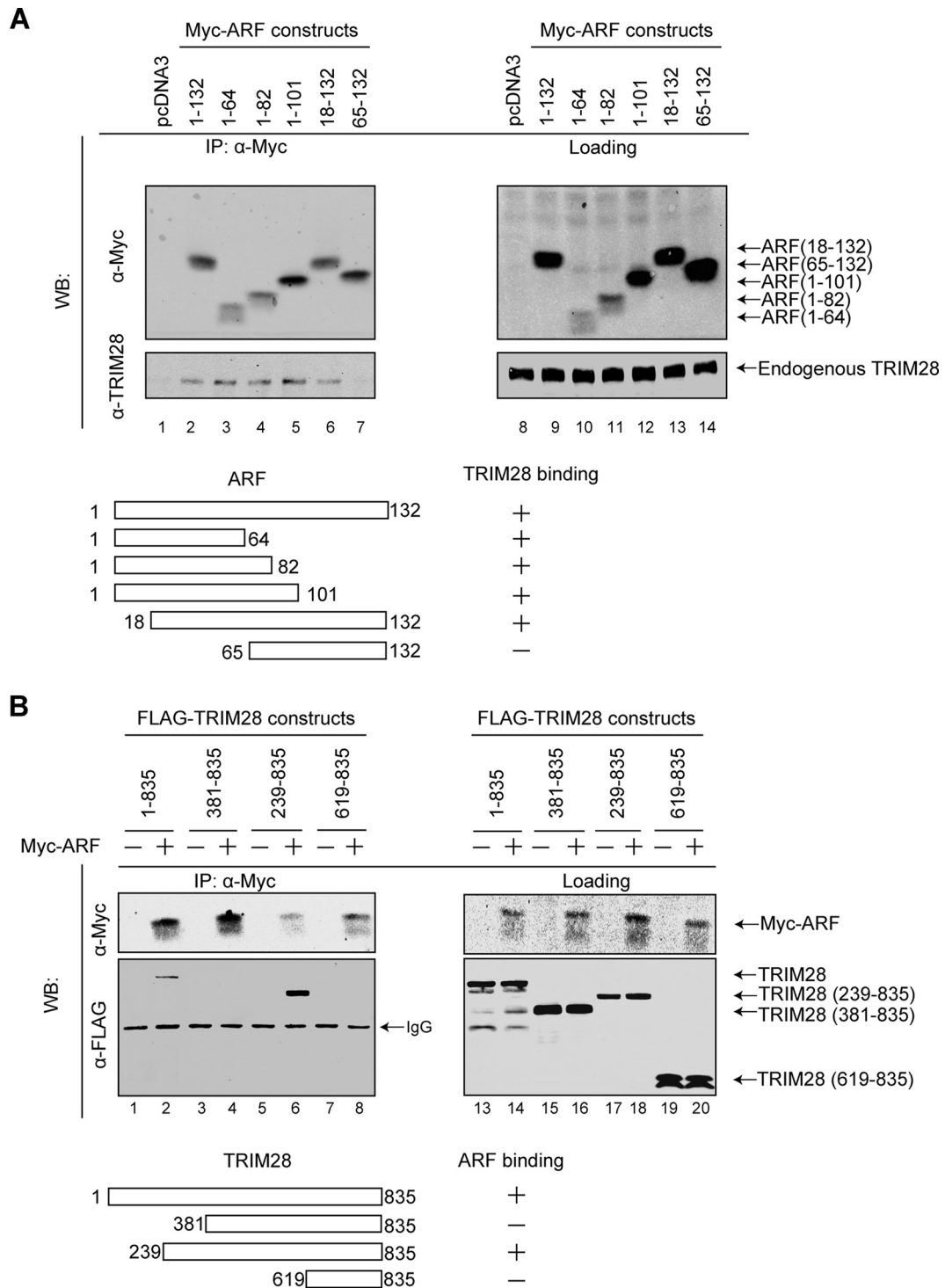


FIG 2 Domain analysis of the ARF-TRIM28 interaction. Mapping of the TRIM28-binding domain of ARF (A) and ARF-binding domain of TRIM28 (B). Lysates of U2OS cells transfected with indicated plasmids were immunoprecipitated with anti-Myc antibody and analyzed by Western blotting (WB) with the indicated antibodies.

knockdown of endogenous TRIM28 reduced the SUMOylation of NPM1 induced by ARF overexpression in H1299 cells (Fig. 3F). Similarly, overexpression of ARF in ARF-negative U2OS cells also increased NPM1 SUMOylation, which was completely attenuated when TRIM28 expression was reduced by siRNA (Fig. 3G),

suggesting that TRIM28 is required for ARF-mediated NPM1 SUMOylation. Further, overexpression of TRIM28 increased NPM1 SUMOylation even in cells that have reduced levels of ARF by shRNA-mediated knockdown (Fig. 3H, lanes 1 and 2), suggesting that TRIM28 could catalyze SUMOylation to some extent

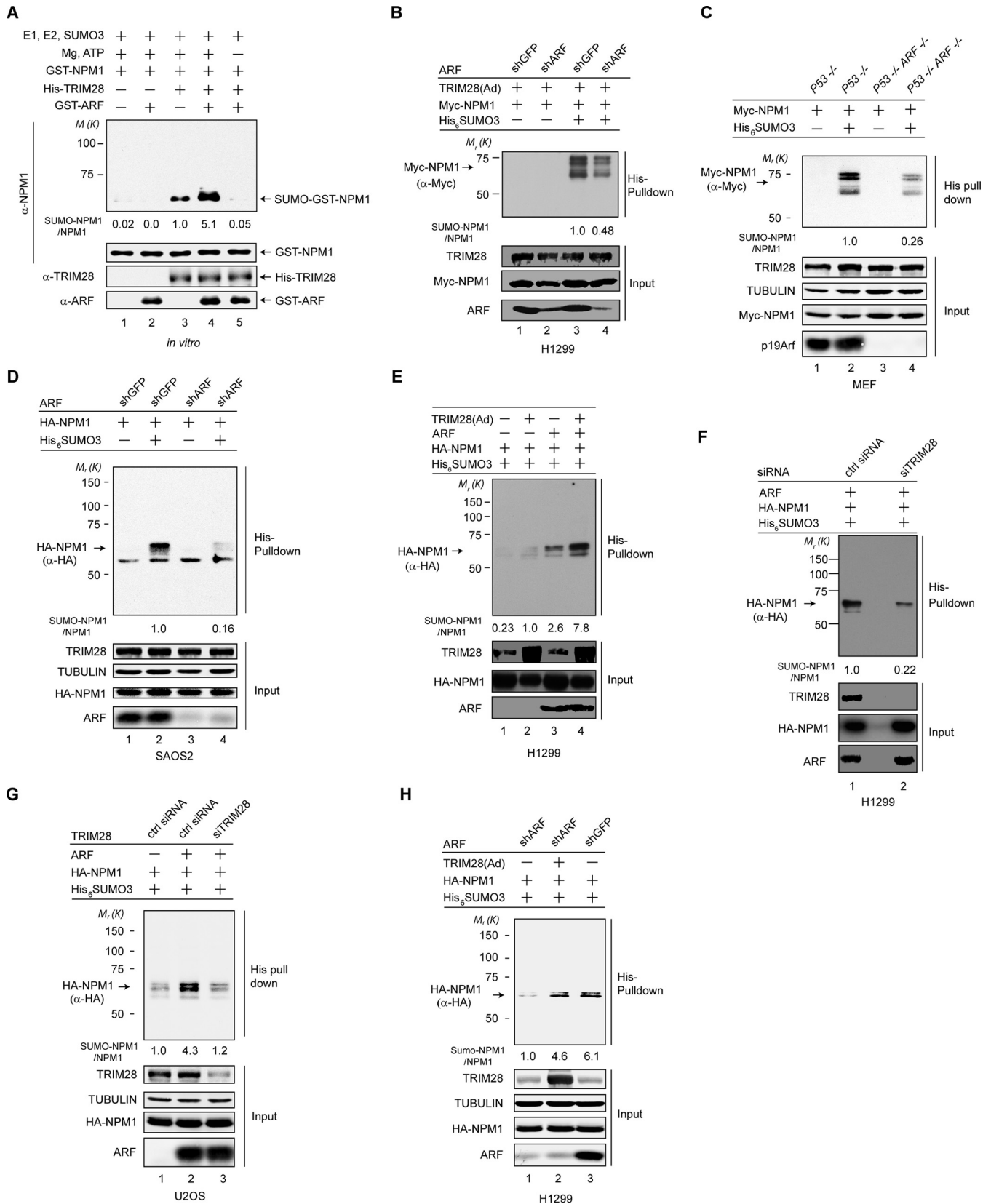


FIG 3 TRIM28 is an E3 ligase for ARF-mediated SUMOylation of NPM1. (A) *In vitro* SUMOylation assay. NPM1 is SUMOylated by TRIM28, and ARF enhances this reaction. The relative band intensities of the SUMOylated NPM1 normalized to non-SUMOylated NPM1 (SUMO-NPM1/NPM1) are shown. The value for lane 3 is set at 1.0. (B) Depletion of ARF reduces SUMOylation of NPM1. Adenovirus (Ad)-encoded TRIM28-infected H1299 cells expressing either GFP or ARF

without the support of ARF, consistent with our data from the *in vitro* SUMOylation assay (Fig. 3A). Collectively, these data suggest that TRIM28 is a SUMO E3 ligase for NPM1 and that ARF may act as a cofactor or E4 SUMO enzyme to enhance TRIM28-mediated NPM1 SUMOylation.

NPM1 can be SUMOylated at lysines 230 and 263 (9, 22). To determine if ARF/TRIM28 induce SUMOylation of these sites, we generated NPM1 point mutants in which these lysines were replaced with arginine (K230R, K263R, and K230R/K263R). We found that NPM mutants were SUMOylated to an extent similar to that of wild-type NPM1 in U2OS cells ectopically expressing ARF (see Fig. S3 in the supplemental material). These data indicate that Lys230 and Lys263 are not responsible for efficient SUMOylation of NPM1 by ARF/TRIM28.

Since we found that NPM1, an ARF-binding protein, is a substrate for TRIM28, we next wanted to test if MDM2, another ARF-binding protein, could also be SUMOylated by TRIM28. It has been shown previously that ARF also enhances MDM2 SUMOylation (4). In contrast with NPM1, TRIM28 overexpression or knockdown did not affect the extent of ARF-mediated MDM2 SUMOylation in H1299 cells (see Fig. S4 in the supplemental material). These data suggest that MDM2 is not a substrate for SUMOylation by TRIM28.

ARF represses centrosome amplification. NPM1 acts as a licensing factor to inhibit centrosome duplication and to coordinate centrosome duplication with DNA replication (23). The lack of NPM1 leads to centrosome amplification and genomic instability (10). NPM1 SUMOylation contributes to its centrosomal localization and to the repression of centrosome duplication (9). If centrosomes duplicate more than once during a cell cycle, centrosome amplification (three or more centrosomes in each cell) occurs, leading to genomic instability. Based on our data (Fig. 3) and a previous report showing that ARF promotes SUMOylation of NPM1 (5), we hypothesized that ARF maintains genome integrity by preventing centrosome amplification through NPM1 SUMOylation. To test this hypothesis, we evaluated the subcellular localization of γ -tubulin in early-passage wild-type and *ARF*^{-/-} MEFs by immunostaining (Fig. 4A, left panel). γ -Tubulin is a major component of the pericentriolar material that surrounds the pair of centrioles in the centrosome and is frequently used as a centrosomal marker (9, 18, 19, 24, 25). We detected a significantly higher percentage of *ARF*^{-/-} MEFs containing three or more centrosomes than of wild-type MEFs (approximately 10% versus 1%, respectively) (Fig. 4A, right panel). These data are consistent with the contribution of endogenous ARF to NPM1 SUMOylation (Fig. 3C). To determine if ARF also regulates centrosome numbers in human cancer cells, we conducted similar

immunostaining experiments in Saos-2 cells (*p53* null). shRNA-mediated knockdown of ARF expression increased the percentage of Saos-2 cells with centrosome amplification (Fig. 4B). The amount of NPM1 SUMOylation was also lower in Saos-2 cells expressing ARF shRNA (Fig. 3D), showing that centrosome amplification and NPM1 SUMOylation status are inversely correlated.

The amplification of centrosomes in *ARF*^{-/-} MEFs was attenuated by the introduction of full-length ARF but not of the ARF deletion mutant ARF(65-132), which lacks the TRIM28-binding region (Fig. 2A and 4C). This observation suggests that the TRIM28-binding region of ARF is critical to prevent centrosome amplification. Introduction of ARF into *p53*^{-/-}; *ARF*^{-/-} MEFs also showed the increase in NPM1 SUMOylation (see Fig. S5A in the supplemental material), suggesting the potential inverse relationship of centrosome amplification and NPM1 SUMOylation in MEFs. Similarly, ectopic expression of ARF but not of the ARF(65-132) deletion mutant enhanced NPM1 SUMOylation (see Fig. S5B in the supplemental material) and attenuated centrosome amplification in ARF-negative U2OS cells (Fig. 4D). These results suggest a role for ARF-mediated NPM1 SUMOylation in the prevention of centrosome amplification.

It has been reported that oncogenic Ras induces centrosome amplification, causing genomic instability (26). Because ARF is induced by oncogenic Ras (1), we tested the idea that ARF may have a tumor-suppressive function by counteracting oncogenic Ras-induced centrosome amplification. We used retroviruses carrying Ha-Ras (V12) mutant to infect early-passage *p53*^{-/-} (*ARF*^{-/-}) or *p53*^{-/-}; *ARF*^{-/-} MEFs. We chose to analyze *p53*^{-/-} and *p53*^{-/-}; *ARF*^{-/-} MEFs since oncogenic Ras does not trigger cellular senescence in these cells, whereas it does so in wild-type MEFs (27). To examine if ARF can counteract Ras-induced centrosome amplification, we stained these MEFs with anti- γ -tubulin. As previously reported, oncogenic Ras significantly induced ARF expression in *p53*^{-/-} MEFs, indicated by Western blotting (1, 7) (Fig. 4E), and we found that the levels of centrosome amplification remained the same regardless of the presence of oncogenic Ras (Fig. 4F). On the other hand, oncogenic Ras significantly induced centrosome amplification in *p53*^{-/-}; *ARF*^{-/-} MEFs (Fig. 4F). These data suggest that the presence of ARF suppresses Ras-induced centrosome amplification. Although p53 has been shown to prevent centrosome amplification (28–30), these data suggest a p53-independent function of ARF to protect cells from oncogenic Ras-mediated centrosome amplification. Taken together, our data suggest that ARF inhibits centrosome amplification.

MEFs from *p53*-null mice have been shown to undergo aneuploidy (31) due to extreme genetic instability and centrosome

shRNA were transfected with the indicated plasmids. SUMOylated proteins were recovered with Ni²⁺-NTA beads and analyzed by Western blotting. The ratio of SUMO-NPM1 to NPM1 is shown as described for panel A. The value of SUMO-NPM1/NPM1 for lane 3 is set at 1.0. (C) Loss of ARF reduces SUMOylation of NPM1. The experiment was performed as described for panel B using early-passage *p53*^{-/-} and *p53*^{-/-}; *ARF*^{-/-} MEFs. The value of SUMO-NPM1/NPM1 for lane 2 is set at 1.0. (D) Depletion of ARF reduces SUMOylation of NPM1. The experiment was performed as described for panel B using Saos-2 cells expressing either GFP or ARF shRNA. The value of SUMO-NPM1/NPM1 for lane 2 is set at 1.0. (E) Overexpression of both TRIM28 and ARF dramatically increases SUMOylation of NPM1. Adenovirus (Ad)-encoded TRIM28-infected H1299 cells were transfected with the indicated plasmids and analyzed as described for panel B. The value of SUMO-NPM1/NPM1 for lane 2 is set at 1.0. (F) Depletion of TRIM28 reduces SUMOylation of NPM1. The experiment was performed as described for panel B using H1299 cells, with the exception that TRIM28 expression was knocked down by siRNA. The value of SUMO-NPM1/NPM1 for lane 1 is set at 1.0. (G) NPM1 SUMOylation induced by overexpression of ARF is reduced by knockdown of TRIM28. The experiment was performed as described for panel B using U2OS cells, with the exception that TRIM28 expression was knocked down by siRNA. The value of SUMO-NPM1/NPM1 for lane 1 is set at 1.0. (H) The reduction of NPM1 SUMOylation by knockdown of ARF is attenuated by overexpression of TRIM28. The experiment was performed as described for panel B using H1299 cells expressing either GFP or ARF shRNA. The value of SUMO-NPM1/NPM1 for lane 1 is set at 1.0.

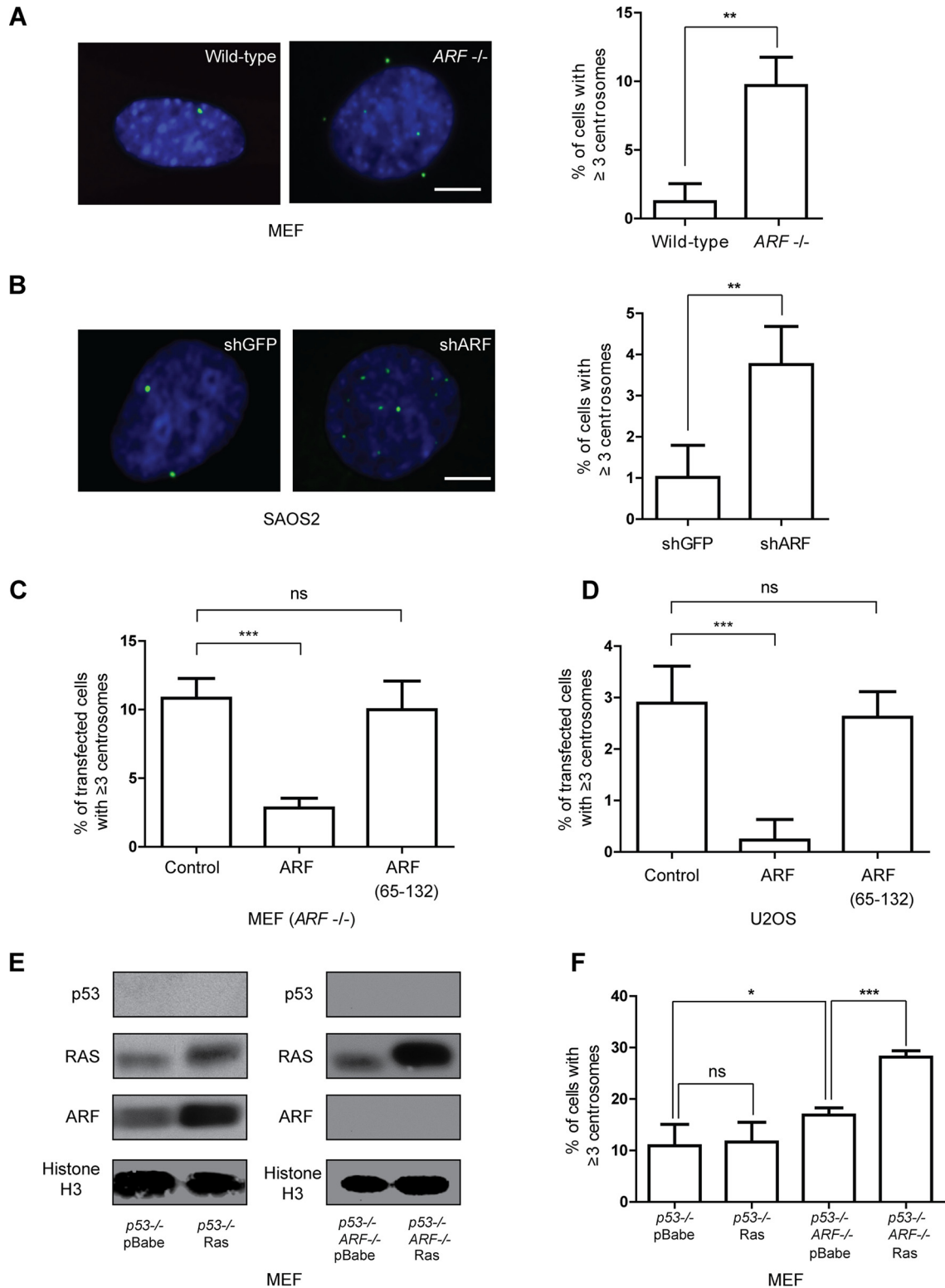


FIG 4 ARF represses centrosome amplification. (A) Loss of ARF results in enhanced centrosome amplification. Representative images (left) of wild-type and early-passage *ARF*^{-/-} MEFs immunostained with anti- γ -tubulin antibody (green) and stained with DAPI (blue). Centrosomes (γ -tubulin-positive foci) were counted and plotted (right). Scale bar, 2 μ m. (B) Depletion of ARF in human cancer cells increases centrosome amplification. Centrosomes in Saos-2 cells expressing either shGFP or shARF were quantified as described for panel A. Scale bar, 2 μ m. (C) Overexpression of ARF but not ARF deletion mutant ARF(65-132), which lacks the TRIM28-binding region, decreases centrosome amplification. *ARF*^{-/-} MEFs were transfected with the indicated plasmids, and centrosomes (γ -tubulin-positive foci) were counted as described for panel A. (D) Overexpression of ARF but not ARF deletion mutant ARF(65-132) decreases centrosome amplification. U2OS cells were transfected with the indicated plasmids, and centrosomes (γ -tubulin-positive foci) were counted as described for panel A. (E and F) Oncogenic Ras-induced centrosome amplification was attenuated by ARF. Early-passage *p53*^{-/-} and *p53*^{-/-}; *ARF*^{-/-} MEFs were infected with empty or (V12) Ras-expressing retroviruses. Cells were selected using puromycin, and cell lysates were harvested and analyzed by Western blotting (E). Histone H3 serves as the loading control. Centrosome numbers were counted as described for panel A (F). Data in panels A to D and F were determined by counting ≥ 200 cells. Data are represented as means \pm standard deviations from three independent experiments. Statistical significance: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant.

amplification (28). It has also been reported that ARF can prevent genomic instability (32) as well as aneuploidy (33, 34) in a p53-independent manner. More recently, *ARF*^{-/-} MEFs (35, 36) and tissue from *ARF*-null mice (35) were shown to exhibit aneuploidy. Therefore, we decided to evaluate chromosome content by metaphase spread analysis using early-passage wild-type, *ARF*^{-/-}, *p53*^{-/-}, and *p53*^{-/-}; *ARF*^{-/-} MEFs. The frequency of aneuploid cells was around 40% for MEFs lacking ARF, 50% for MEFs lacking p53, 80% for MEFs lacking both ARF and p53, and only around 10% for wild-type MEFs (see Fig. S6 in the supplemental material). These observations support a p53-independent role for ARF in maintaining chromosome stability and are consistent with the observed centrosome amplification in *ARF*^{-/-}, *p53*^{-/-}, and *p53*^{-/-}; *ARF*^{-/-} MEFs compared to wild-type MEFs (Fig. 4A and F).

TRIM28 contributes to ARF-mediated repression of centrosome amplification. To further understand how ARF represses centrosome amplification, we examined TRIM28 as a potential link between ARF and NPM1 in this process. We showed that ARF binds TRIM28 (Fig. 1) and that ARF-induced NPM1 SUMOylation was significantly attenuated by TRIM28 knockdown (Fig. 3F and G). To determine if TRIM28 contributes to ARF-mediated inhibition of centrosome amplification, we knocked down endogenous TRIM28 expression with siRNA in Saos-2 (*p53*-null) cells expressing either control shGFP or shARF and determined the number of centrosomes per cell (Fig. 5A). The knockdown efficiency of ARF or TRIM28 was confirmed by Western blotting (see Fig. S7 in the supplemental material). Knockdown of either ARF or TRIM28 alone significantly increased the percentage of cells having three or more centrosomes compared to control (Fig. 5A), and this percentage slightly increased when both genes were simultaneously reduced (Fig. 5A). Since knockdown of each gene was not complete, it is difficult to say if ARF and TRIM28 double knockdown has a synergistic effect. To provide further support for the tight collaboration of ARF and TRIM28, we restored the expression of ARF in shARF Saos-2 cells with either full-length ARF or the ARF(65-132) deletion mutant, which cannot bind to TRIM28. We observed reduced centrosome amplification when full-length ARF, but not ARF(65-132), was introduced (Fig. 5B, lanes 2 and 4). This suppression observed with full-length ARF was attenuated by siRNA-mediated depletion of TRIM28 (Fig. 5B, lane 3). These results are consistent with the hypothesis that TRIM28 is required for ARF-mediated inhibition of centrosome amplification. In addition, overexpression of TRIM28 reduced centrosome amplification in Saos-2 cells (Fig. 5C, lanes 3 and 4) and in shARF-expressing Saos-2 cells, which express reduced levels of ARF (Fig. 5C, lanes 1 and 2). These findings provide further support that TRIM28 itself can catalyze NPM1 SUMOylation to some extent (Fig. 3A and H), thereby suppressing centrosome amplification, and that ARF enhances the ability of TRIM28 to SUMOylate NPM1 and inhibits centrosome amplification.

Since ARF is known to bind to NPM1, we examined whether ARF, TRIM28, and NPM1 can form a ternary complex, thereby promoting SUMOylation of NPM1. However, we could not detect a stable interaction between TRIM28 and NPM1 in the absence or presence of ARF by immunoprecipitation (see Fig. S8 in the supplemental material), despite the observation that TRIM28 can SUMOylate NPM1 *in vitro* (Fig. 3A). This could be due to the limitation of detecting short-lived, transient binding, such as the interaction between an enzyme and its substrate.

To determine whether the E3 SUMO ligase activity of TRIM28 is required to suppress centrosome amplification, we generated the TRIM28 mutant C651F, which abolishes this enzymatic activity (11). Either wild-type or mutant TRIM28 was transfected into NIH 3T3 (ARF-negative) cells, and the expression levels of wild-type and mutant TRIM28 (C651F) were similar (Fig. 5D). We observed that expression of wild-type TRIM28 but not of the TRIM28 C651F mutant increased NPM1 SUMOylation in the presence or absence of ARF (Fig. 5D; see also Fig. S9 in the supplemental material), confirming that TRIM28 mutant C651F loses its E3 SUMO ligase activity. The levels of NPM1 SUMOylation were higher in cells with coexpression of wild-type TRIM28 and ARF than in cells with ARF overexpression alone (Fig. 5D, lanes 2 and 4) or in cells with TRIM28 overexpression alone (see Fig. S9, lanes 2 and 5, in the supplemental material). These results suggest that TRIM28 and ARF cooperate to enhance NPM1 SUMOylation dependent on the E3 ligase activity of TRIM28. We also confirmed that ARF and TRIM28 interact in NIH 3T3 cells (see Fig. S10 in the supplemental material). Under these conditions, we observed that expression of wild-type but not mutant TRIM28 reduced the percentage of NIH 3T3 cells with three or more centrosomes in the absence of ARF (Fig. 5E, lanes 1, 2, and 3). With the support of ARF, wild-type but not mutant TRIM28 further reduced the percentage of NIH 3T3 cells with three or more centrosomes (Fig. 5E, compare columns 4, 5, and 6), suggesting that the E3 SUMO ligase activity of TRIM28 is required for suppression of centrosome amplification.

NPM1 is reported to localize to unduplicated centrosomes (23). Since it was shown that NPM1 SUMOylation is required for centrosomal localization of NPM1 and suppression of centrosome duplication (9), we next explored whether coexpression of TRIM28 and ARF affects the subcellular localization of NPM1. We transfected NIH 3T3 cells with FLAG-TRIM28 and ARF and coimmunostained the cells with anti- γ -tubulin and anti-NPM1 (PabN1). The PabN1 NPM1 antibody is known to react with only mouse NPM1 and recognizes centrosomal but not nucleolar NPM1 (18, 19). We found that expression of wild-type TRIM28 but not mutant TRIM28 C651F increased the frequency of cells with NPM1 localization in the centrosome (Fig. 5F, lanes 1, 2, and 3). In addition, coexpression of ARF with wild-type TRIM28 but not with mutant TRIM28 further increased the frequency of cells with NPM1 localization in the centrosome (Fig. 5F, lanes 4, 5, and 6, and G), suggesting that the E3 SUMO ligase activity of TRIM28 contributes to the centrosomal localization of NPM1. Taken together, these findings demonstrate that ARF enhances NPM1 SUMOylation via TRIM28, which contributes to increased centrosomal localization of NPM1 and inhibits centrosome amplification (Fig. 6).

DISCUSSION

ARF has been known to have p53-independent functions for tumor suppression (2). One such function is the ability of ARF to promote SUMOylation. However, ARF does not possess the E3 SUMO ligase activity necessary to catalyze the SUMOylation reaction *in vitro* (6), and the E3 SUMO ligase responsible for ARF-mediated SUMOylation was not known. Thus, the mechanism and physiological relevance of ARF-induced SUMOylation were unclear. In this study, we report for the first time that ARF directly interacts with the nuclear protein TRIM28 and that TRIM28 is an E3 SUMO ligase for ARF-mediated SUMOylation of NPM1.

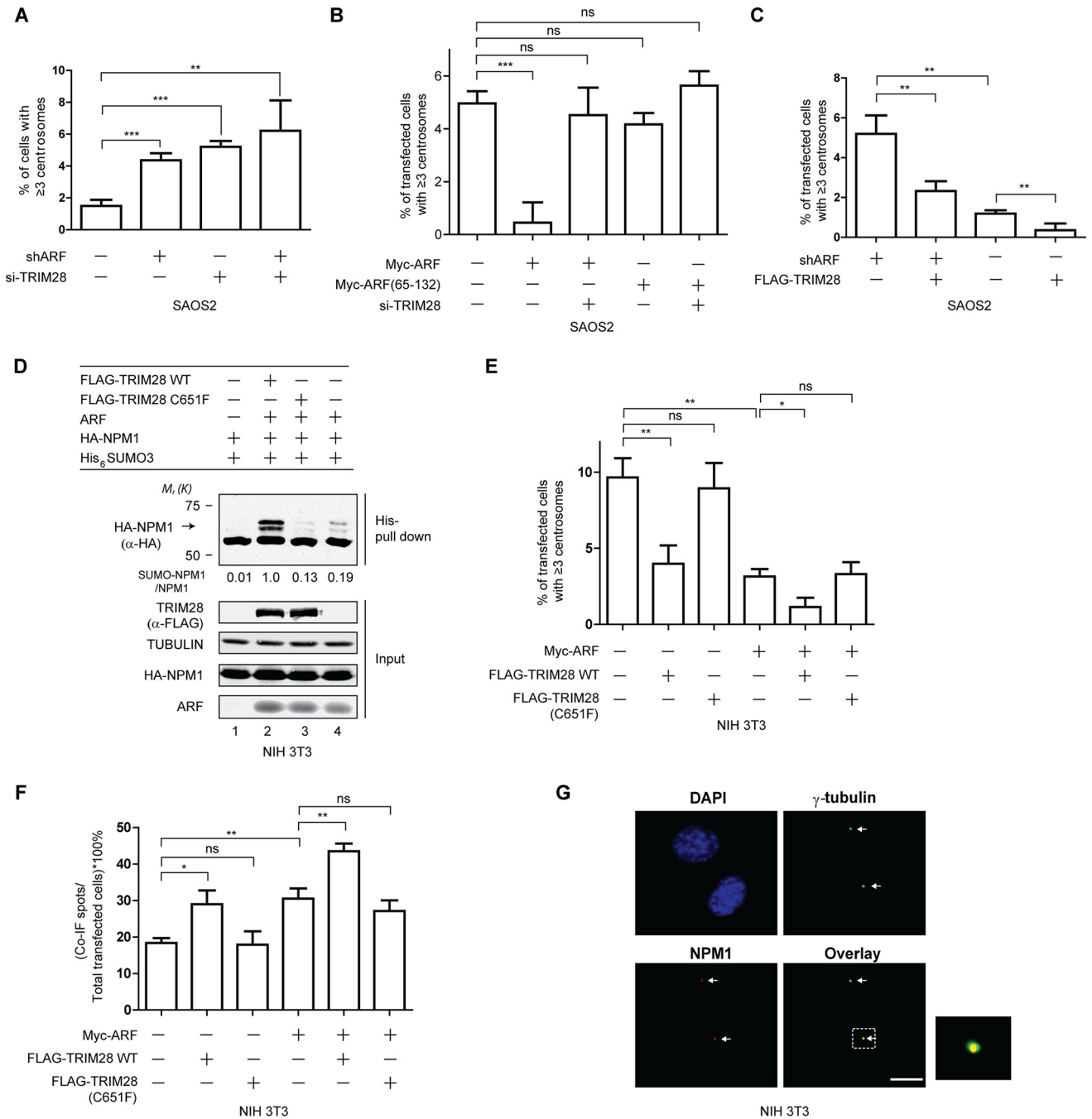


FIG 5 TRIM28 contributes to ARF-mediated repression of centrosome amplification. (A) TRIM28 and ARF contribute to inhibition of centrosome amplification. Saos-2 cells expressing shGFP or shARF were transfected with TRIM28 siRNA and stained with anti- γ -tubulin. Centrosome numbers were counted as described for Fig. 4. (B) Reduction of centrosome amplification by ARF overexpression is attenuated by knockdown of TRIM28. Saos-2 cells expressing shARF were transfected with the indicated plasmids and siRNA and stained with anti- γ -tubulin. Centrosome numbers were counted as described for Fig. 4. (C) TRIM28 contributes to the reduction of centrosome amplification even in shARF-expressing cells. Saos-2 cells expressing shGFP or shARF were transfected with FLAG-TRIM28 expressing plasmid and stained with anti- γ -tubulin. Centrosome numbers were counted as described for Fig. 4. (D) Wild-type TRIM28, but not a catalytic dead mutant TRIM28 C651F, enhances SUMOylation of NPM1 in the presence of ARF. NIH 3T3 cells were transfected with the indicated plasmids, recovered with Ni²⁺-NTA beads, and analyzed by Western blotting. The relative band intensities of the SUMOylated NPM1 normalized to non-SUMOylated NPM1 (SUMO-NPM1/NPM1) are shown. The value for lane 2 is set at 1.0. (E) ARF and TRIM28 reduce centrosome amplification dependent on E3 SUMO ligase activity of TRIM28. NIH 3T3 cells were cotransfected with the indicated plasmids and stained with anti- γ -tubulin. Centrosome numbers were determined as described for Fig. 4. (F and G) ARF and TRIM28 enhance centrosomal translocation of NPM1 dependent on E3 SUMO ligase activity of TRIM28. NIH 3T3 cells were transfected with the indicated plasmids, and colocalization of NPM1 and γ -tubulin was assessed by immunostaining. The percentage of NPM1 colocalized with γ -tubulin in transfected cells was plotted (F); representative images of colocalization between NPM1 and γ -tubulin are shown (G). Arrows indicate the positions of centrosomes. Scale bar, 2 μ m. The field enclosed by white dashed lines is shown at a higher magnification to the right of merged images. More than 200 cells in panels A, B, and C and more than 300 cells in panels E and F were counted. Data are represented as means \pm standard deviations from three independent experiments. Statistical significance: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant.

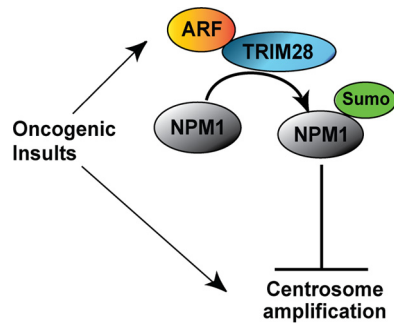


FIG 6 Model of ARF-mediated NPM1 SUMOylation in preventing centrosome amplification. ARF prevents centrosome amplification induced by oncogenic insults by enhancing SUMOylation of NPM1 via TRIM28. The ARF-TRIM28 interaction promotes NPM1 SUMOylation, enhancing its translocation to centrosomes, thereby preventing centrosome amplification induced by oncogenic insults.

TRIM28 has been demonstrated to be an E3 SUMOylation ligase toward itself (11), IRF7 (12), and Vps34 (13). Our data indicated that some SUMOylated NPM1 was still observed despite the efficient knockdown of TRIM28 expression (Fig. 3F), suggesting that TRIM28 may not be the sole E3 SUMO ligase for NPM1 and/or that other E3 SUMO ligases compensate for reduced TRIM28 expression. Nevertheless, NPM1/B23 SUMOylation induced by ARF overexpression in U2OS cells (ARF negative) was completely attenuated by TRIM28 knockdown (Fig. 3G), suggesting that TRIM28 is critical at least for NPM1 SUMOylation induced by ARF. We also found that MDM2, another ARF-binding protein, was not SUMOylated by TRIM28 (see Fig. S4 in the supplemental material). The contribution of TRIM28 to SUMOylation of other ARF-binding proteins such as E2F-1 and WRN remains to be explored.

Centrosome duplication is a highly coordinated process to ensure genomic integrity. Deregulation of the duplication machinery leads to centrosome amplification, which contributes to the development of cancer (37, 38). It is well established that different posttranslational modifications of NPM1 affect its localization to the centrosome and, in turn, its essential role in regulating centrosome duplication. For example, Cdk2/cyclin E-mediated phosphorylation of NPM1 (Thr199) induces dissociation of NPM1 from centrosomes, followed by initiation of centrosome duplication (19, 23). Conversely, SUMOylation of NPM1 enhances its centrosomal localization (9). The localization of NPM1 to the centrosome is essential to inhibit undesired centrosome duplication (9, 19, 23), and NPM1 knockout MEFs were reported to exhibit unrestricted centrosome duplication, thereby leading to centrosome amplification and genomic instability (10). These reports are consistent with our findings that the enhanced localization of NPM1 to centrosomes, via its increased SUMOylation by ARF and TRIM28, inhibits centrosome amplification.

Centrosome amplification is frequently observed in many cancer types (39, 40), and it also causes cellular invasion (41). Oncogenic Ras (26), c-Myc (26), Aurora A (42), activating mutant epidermal growth factor receptor (EGFR) (43), and oncogenic Met (44) have been shown to induce centrosome amplification. Our study suggests a potential role for ARF in preventing centrosome amplification mediated by oncogenic insults. ARF is usually expressed at very low levels, and a major tumor-suppressive role of

ARF serves to counteract oncogenic insults. We observed that oncogenic Ras efficiently induced centrosome amplification in $p53^{-/-}$; $ARF^{-/-}$ MEFs but not in $p53^{-/-}$ MEFs, in which ARF was induced by oncogenic Ras (Fig. 4E and F). These data support a physiological tumor-suppressive role of ARF in suppressing Ras-induced centrosome amplification at least in MEFs (Fig. 6). However, given that ARF has many p53-independent interacting partners, whether ARF represses Ras-mediated transformation by preventing centrosome amplification is still unclear and remains to be elucidated *in vivo*.

The association between the loss of p53 and centrosome amplification has been demonstrated previously (28). p53 can prevent centrosome amplification in a transcriptionally dependent (29) or independent manner (30), although the underlying mechanisms remain poorly understood. Since it has been shown that p53 inactivation alone is insufficient to induce aneuploidy in the case of human diploid cells (45), it is likely that p53-independent factors are also involved in guarding the centrosome duplication process and maintaining chromosome stability. For instance, RABL6A, a binding partner of ARF, was recently demonstrated to control centrosome amplification (46). As ARF activates p53, it is possible that ARF prevents centrosome amplification induced by oncogenic insults by both p53-dependent and -independent pathways. An earlier study indicated that there are three key residues in ARF (Val²⁴, Thr³¹, and Ala⁴¹) that are critical for mediating a p53-independent role in chromosomal stability (33). Interestingly, these residues lie within the TRIM28-binding domain of ARF (Fig. 2A). The novel tumor-suppressor activity of ARF in maintaining genome integrity revealed in this study underscores the importance of ARF in cancer prevention and provides new insight into the p53-independent tumor suppression function of ARF.

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S.H.N., Y.I., J.A., and K.I. performed the experiments. K.T. performed mass spectrometry analysis. M.K., A.K.G., and S.H.L. performed metaphase spread analysis. S.H.N., Y.I., and K.I. designed the experiments and wrote the manuscript.

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