Sumoylation induced by the Arf tumor suppressor: A p53-independent function

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The mouse p19^{Arf} protein has both p53-dependent and p53-independent tumor-suppressive activities. Arf triggers sumoylation of many cellular proteins, including Mdm2 and nucleophosmin (NPM/B23), with which p19^{Arf} physically interacts *in vivo*, and this occurs equally well in cells expressing or lacking functional p53. In an Arf-null NIH 3T3 cell derivative (MT-Arf cells) engineered to reexpress an Arf transgene driven by a zinc-inducible metallothionein promoter, sumoylation of endogenous Mdm2 and NPM proteins was initiated as p19^{Arf} was induced and was observed before p53-dependent cell cycle arrest. Predominately nucleoplasmic molecules visualized by immunofluorescence with antibodies to small ubiquitin-like modifier (SUMO) 1 localized to nucleoli as p19^{Arf} accumulated there. Two Arf mutants, one of which binds to Mdm2 and NPM but is excluded from nucleoli and the other of which enters nucleoli but is handicapped in binding to Mdm2 and NPM, were defective in inducing sumoylation of these two target proteins and did not localize bulk sumoylated molecules to nucleoli. The CELO adenovirus protein, Gam1, which inhibits the SUMO activating enzyme (E1) and leads to down-regulation of the SUMO conjugating enzyme (E2/Ubc9), had no overt effect on the ability of p19^{Arf} to activate p53 or the p53-responsive genes encoding Mdm2 and p21^{Cip1}, despite the fact that Arf-induced sumoylation of Mdm2 was blocked. Reduction of Ubc9 levels with short hairpin RNAs rendered similar results. We suggest that Arf's p53-independent effects on gene expression and tumor suppression might depend on Arf-induced sumoylation.

Gam1 | Ubc9

The mouse *Ink4a-Arf* locus encodes two tumor-suppressor proteins, p16^{Ink4a} and p19^{Arf} (p14^{ARF} in humans) that increase the growth-suppressive activities of the retinoblastoma-family proteins (Rb, p107, and p130) and the p53 transcription factor, respectively (1–3). By inhibiting Cdk4 and Cdk6, p16^{Ink4a} helps to maintain Rb-family proteins in their active hypophosphorylated state, thereby inhibiting an E2F-dependent transcriptional program that is required for cellular DNA replication. In contrast, p19^{Arf} antagonizes the p53 negative regulator Mdm2 (Hdm2 in humans) to trigger a p53 transcriptional response that can lead to proliferative arrest or apoptosis (4). Expression of *Ink4a* and *Arf* is regulated by distinct promoters upstream of alternative first exons whose products are spliced to a common second exon translated in alternative reading frames (from which *Arf* gets its name) (2). The two genes are induced by different stress signals and can be separately mutated or silenced in tumor cells (4).

Arf is activated by abnormally elevated and sustained mitogenic signals triggered by oncogenes but not by physiologic signaling thresholds conveyed by their appropriately regulated protooncogenic counterparts (4). For example, *Arf* is not induced by Myc or Ras during normal cell-cycle progression, but it is transcribed when such signals are constitutively enforced through Myc translocation or oncogenic Ras mutation. By activating p53, *Arf* serves to eliminate cells sustaining such mutations. However, *Arf* is not directly induced by DNA damage signals that trigger p53 activity through other signal-transduction

pathways (3), although its loss can modify the damage response (5). In this sense, *Arf* serves as a fuse that gates mitogenic current, preventing abnormal cell proliferation in response to oncogene activation.

Although it is generally accepted that much of *Arf*'s tumor-suppressor activity is mediated through p53, *Arf* also has p53-independent functions. Enforced expression of p19^{Arf} can arrest the proliferation of p53-null cells, although much less efficiently than in cells that retain WT p53 (6). Primary mouse fibroblasts and B lymphocytes lacking both *Arf* and *p53* grow faster in culture than do cells lacking only one of the two genes (3, 7), and mice lacking *Arf*, *Mdm2*, and *p53* are much more prone to developing cancer than are mice lacking *Mdm2* and *p53* (8). Arf inhibits ribosomal RNA processing (9), interacts with topoisomerase I (10), and mediates p53-independent effects on gene expression by negatively regulating other transcription factors such as Btk coactivators (11), E2Fs (12–15), Myc (16, 17), and NFκB (18). These p53-independent activities of Arf are relatively poorly understood.

Enforced expression of p14^{ARF} in human cells promotes sumoylation of several cotransfected cellular proteins, including Hdm2 (19, 20), the Werner helicase (WRN) (21), and the transcription factors E2F-1 and HIF-1α (22). Covalent addition of the small ubiquitin-like modifiers (SUMO-1, SUMO-2, and SUMO-3) to the ε-amino groups of certain lysine residues in protein substrates is carried out by a single E1 enzyme (the SUMO-1-activating enzyme 1/2 heterodimer also known as Aos1/Uba2) and a single E2 enzyme (Ubc9) in a process analogous to ubiquitination (23–25). Before conjugation, SUMO isoforms must be cleaved by a SUMO-specific protease to generate a C-terminal diglycine motif. Processed SUMO can then be activated in an ATP-dependent manner to form a thioester bond between SUMO and the catalytic cysteine of the E1. The E1 mediates SUMO transfer to a cysteine residue in Ubc9, after which one of several E3s can interact with both Ubc9 and protein substrates to accelerate the rate and magnitude of their sumoylation (26–28). Mammalian protein inhibitor of activated signal transducer and activator of transcription (PIAS) proteins appear to act as SUMO E3 ligases for LEF1, p53, and c-Jun, whereas the nucleoporin RanBP2 facilitates sumoylation of RanGAP1, Sp100, histone deacetylase (HDAC) 4, and Mdm2 (24, 25, 28). SUMO-specific proteases serve a dual role in the SUMO cascade, being required not only for initial SUMO processing but also for cleaving ligated SUMO from protein substrates (29–31). Unlike ubiquitin, SUMO-1 does not polymerize, so the formation of “ladders” of modified proteins is presumed to represent monosumoylation of multiple lysines. However, SUMO-2 and SUMO-3 have internal SUMO modification sites that allow formation of polymeric chains.

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Abbreviations: HDAC, histone deacetylase; NPM, nucleophosmin; WRN, Werner helicase; IRES, internal ribosome entry site; MSCV, murine stem cell virus; SUMO, small ubiquitin-like modifier; MT, metallothionein promoter; MEF, mouse embryo fibroblast.

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Effects of sumoylation are highly diverse and can influence protein transport, modulation of gene expression (usually, but not always, down-regulation), ubiquitination, DNA repair, and centromeric chromatid cohesion (24, 25, 32, 33). Here, we propose that the p53-independent tumor-suppressive effects of p19^{Arf} may be mediated by its ability to enhance sumoylation of a diverse group of protein targets.

Materials and Methods

Cells and Culture Conditions. Human 293T cells, mouse NIH 3T3 fibroblasts, and zinc-inducible metallothionein promoter-containing (MT)-Arf cells (11) were cultured in DMEM (Cambrex, Walkersville, MD) supplemented with 10% FCS (HyClone), 4 mM glutamine, and 100 units each of penicillin and streptomycin. Primary mouse embryo fibroblasts (MEFs) of various genotypes (8) were cultured as described in ref. 34.

Expression Vectors. cDNAs encoding C-terminally processed forms of SUMO were amplified by reverse transcription and PCR using 1 μ g of RNA template from 293T cells, Moloney murine leukemia virus RT (Invitrogen), and HotStarTaq DNA polymerase (Qiagen, Valencia, CA) with the following primers: SUMO-1 (sense), 5'-gcgggatcctctgaccaggaggcaaaccttcaactg-3'; (antisense), 5'-gcgctcgagctaaccctccgttctgtctgataaac-3'; SUMO-2 (sense), 5'-gcgggatcgcgcgacgaaagcccaaggaggagtc-3'; (antisense), 5'-gcgctcgagctaaccctccgttctgtctggaacac-3'; SUMO-3 (sense), 5'-gcgggatcctccgaggagaagcccaaggagggtgtg-3'; (antisense), 5'-gcgctcgagctaaccctccgttctgtctggaacac-3'. Products cloned into pGEM-T Easy (Promega) were isolated with BamHI and XhoI and cloned into pcDNA3 (Invitrogen) harboring an engineered His₆ tag constructed by insertion of the following annealed oligonucleotides into the pcDNA3 KpnI and XhoI sites: (sense), 5'-cgccaccatggtcatcatcctcatcatggtg-3' and (antisense) 5'-gatccaccatgatgatgatgatgatgagccatggtg-cggtac-3'. pcDNA3-His₆ SUMO-1 was digested with KpnI, blunted with DNA polymerase II Klenow fragment, and digested with XhoI to release the fragment encoding His₆-SUMO-1. This fragment was ligated into a murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-GFP vector (8) that had been digested with EcoRI, blunted as above, and digested with XhoI. pcDNA3-HA-Arf (HA, hemagglutinin) and pcDNA-Mdm2 were made previously (35). DNA fragments encoding HA-Arf, HA-Arf Δ 2-14, and HA-Arf Δ 26-37 (36) were isolated from pSRa-MSV-TKneo (37) by digestion with EcoRI and were recloned into the EcoRI sites of pcDNA3 and MSCV-IRES-PURO (8). pSG9M-myc-Gam1 and pSG9M-myc-Gam1 mutant (258L, 265A) (38) were used for transfection. The cDNAs were amplified by PCR with the following primers: (sense), 5'-tttgatcatggaacaaaactcatctc-3'; (antisense), 5'-tttctcgagttacagagaatgtagg-3'. After digestion with EcoRV and XhoI, purified fragments were recloned into the SnaBI and XhoI sites of MSCV-IRES-PURO. Cells infected with MSCV-IRES-PURO viruses were not selected in puromycin. Ubc9 short hairpin RNA was generated from the following annealed primers: (sense), 5'-gatccccgggaaggaggctgttcaattcaagagattgaacaagcctcttc-3'; (antisense), 5'-agcttttccaaaagggaaggaggctgttcaattcttgaattgaacaagc-3'. These annealed primers were inserted into the BglII and HindIII sites of pSUPER.retro.puro (Oligoengine, Seattle).

Vector Transduction and Protein Detection. Cotransfection of expression plasmids into 293T cells was performed by using FuGENE 6 (Roche, Indianapolis) according to the manufacturer's instructions. Mouse cells were infected at >90% efficiency with MSCV-IRES-GFP or MSCV-IRES-PURO vectors encoding genes of interest under control of the MSCV promoter. Cells harvested 3 days later were resuspended in lysis buffer containing protease inhibitors; proteins in centrifuged lysates were quantified by bicinchoninic acid assay (Pierce), electrophoretically

Fig. 1. Arf-induced sumoylation of Mdm2, NPM, and other cellular proteins. (A) After cotransfection of expression plasmids encoding p19^{Arf} and His₆-SUMO-1 into 293T cells (as indicated at the top), proteins from cell lysates affinity-purified on nickel resin in buffer containing 8 M urea were electrophoretically separated on denaturing gels containing SDS and were immunoblotted with antibody to SUMO-1. The positions of marker proteins of known molecular masses (kDa) are indicated on the left of A (and, similarly, in subsequent figures). The bracket indicates the positions of sumoylated proteins. (B) Proteins from cells cotransfected with the indicated expression plasmids (top) were resolved as above and were blotted with antibodies to Mdm2 (Upper) and NPM (Lower). Based on mass, the fastest-migrating NPM species appears to represent the monosumoylated form.

cally separated on polyacrylamide gels containing SDS, transferred to poly(vinylidene difluoride) membranes (Millipore), and detected, all as described in ref. 37, with 5C3 mAb to p19^{Arf} (39), Mdm2 (mAb 2A10), NPM (32-5200, Zymed), Myc tagged-Gam1 (MAb 9E10), p53 (FL-393), p21^{Cip1} (C-19), and Ubc9 (H-81) (the latter three from Santa Cruz Biotechnology). For purification of His₆-tagged sumoylated proteins, 10% of a trypsinized cell suspension was taken for direct protein immunoblotting. The remaining cells were resuspended in lysis buffer containing 8 M urea, and His₆-tagged proteins recovered with nickel beads (Qiagen) were eluted with imidazole, diluted with sample buffer, and separated on gels containing SDS as described in ref. 37. After transfer to membranes, proteins were immunoblotted with antibodies to SUMO-1 (33-2400, Zymed), Mdm2, and NPM.

Results

Sumoylation Induced by Mouse p19^{Arf}. Plasmids encoding p19^{Arf} and His₆-SUMO-1 were cotransfected into human 293T cells, and His-tagged proteins recovered on nickel beads in the presence of 8 M urea were electrophoretically separated on denaturing gels. Immunoblotting with antibodies to SUMO-1 revealed that introduction of p19^{Arf} greatly increased net protein sumoylation (Fig. 1A). 293T cells do not express active p53 and do not undergo cell cycle arrest when transfected with p19^{Arf}, implying that neither p53 nor perturbations in cell-cycle progression are essential for this process. Immunoblotting of recovered His-tagged proteins with antibodies to Mdm2 and NPM, two proteins that physically interact with p19^{Arf}, demonstrated that both could be multisumoylated, regardless of which SUMO isoform was used (Fig. 1B). The mobilities and spacing of these protein species, particularly obvious for NPM (unmodified molecular mass = 37 kDa), and the similar ladders obtained by using SUMO-1, SUMO-2, and SUMO-3 argue for sumoylation at multiple sites. Although sumoylated lysine residues often are found within ψ KXE consensus sequences, where ψ is an aliphatic residue (26, 31), mutation of three such consensus sequences in NPM (lysines 24, 32, and 54) alone or in combination did not eliminate sumoylation (data not shown), consistent with studies performed elsewhere with p14^{ARF} and WRN (21). Cotransfection of p19^{Arf} together with His₆-SUMO-1 and expression plasmids encoding a number of other proteins that do not bind

Fig. 2. Induction of sumoylation in inducible MT-Arf cells. (A) MT-Arf cells and parental Arf-null NIH 3T3 infected with a retroviral vector encoding His₆-SUMO1 were induced with zinc sulfate for the times indicated at the top. Total cellular proteins separated on denaturing gels were transferred to a membrane and blotted with antibodies to the proteins indicated at the left. (B) Proteins in the same cell lysates adsorbed to nickel beads were recovered, separated on denaturing gels, and blotted with antibodies to Mdm2 (Upper) and NPM (Lower). NPM is basally monosumoylated in NIH 3T3 cells (left lanes). (C) Cells treated with zinc (or not) (indicated at left) to induce p19^{Arf} (Lower) or not (Upper) were immunostained with antibodies to p19^{Arf} and SUMO-1 as indicated at the top. Arf localized SUMO-1-stained molecules to nucleoli (Lower).

directly to p19^{Arf} failed to show sumoylation of two other nucleolar proteins (ribosomal protein L7 and fibrillarin), three other known SUMO targets (HDAC-1 and HDAC-2, and Ran-GAP1), or p53 (negative data not shown). Although p53 was previously reported to undergo p14^{ARF}-dependent sumoylation, this required cointroduction of Mdm2, which can bind to both p14^{ARF} and p53 simultaneously (20). Therefore, it appears that a physical interaction between p19^{Arf} and selected target proteins is necessary for their efficient sumoylation.

Timing of Arf-Induced Sumoylation and Localization of Sumoylated Molecules to Nucleoli. We next studied Arf-induced sumoylation of Mdm2 and NPM in MT-Arf cells, an Arf-null NIH 3T3 cell derivative into which we had previously introduced an integrated Arf transgene regulated by the zinc-inducible metallothionein promoter (11). MT-Arf cells infected with a retrovirus encoding processed His₆-SUMO-1 were treated 3 days later with 80 μ M zinc for various periods, and cellular proteins were separated on denaturing gels and immunoblotted with antibodies to p19^{Arf}, p53, and to both Mdm2 and p21, which are encoded by canonical p53-responsive genes (Fig. 2A). Importantly, the levels of p19^{Arf} expressed in induced MT-Arf cells approximate those observed in primary MEFs undergoing replicative senescence. Arf was first detected after 4 h of zinc treatment and achieved high levels by 8 h, well before most MT-Arf cells exited the cell cycle (at 20–24 h; refs. 9 and 11). As expected, p53 was stabilized, and Mdm2 and p21^{Cip1} were induced with similar kinetics (Fig. 2A).

His-tagged proteins affinity-purified from the same cells under denaturing conditions in 8 M urea were separated and immunoblotted with antibodies to Mdm2 and NPM (Fig. 2B). Sumoylation of Mdm2 was detected as the protein was induced and increased throughout the duration of the experiment (Fig. 2B Upper), even though parallel increases in total Mdm2 protein were not seen at the later time points (Fig. 2A). Although most Mdm2 protein migrated as a characteristic doublet, minor and more slowly migrating species (Figs. 2A and 4B) corresponded in their mobilities to sumoylated forms. NPM, which is a very abundant nucleolar protein, was sumoylated in Arf-null NIH 3T3 cells, probably at only one site based on the apparent molecular mass of the single modified protein species. NPM monosumoylation was seen in uninduced MT-Arf cells, but NPM sumoylation increased after p19^{Arf} induction, and multisumoylated species were detected at later times.

Most p19^{Arf} in cells resides within high-molecular-mass complexes within the nucleolus (2), where many additional Arf-interacting proteins reside (40). Immunofluorescence analyses performed with antibodies to p19^{Arf} and SUMO-1 revealed that although most of the SUMO-1 signal was dispersed throughout the nucleoplasm in WT MEFs (data not shown) or in NIH 3T3 cells that lack the Arf gene, induction and nucleolar accumulation of p19^{Arf} in MT-Arf cells localized SUMO-1-containing molecules to nucleoli (Fig. 2C).

Arf Mutants Defective for Sumoylation. We next studied the effects of two previously characterized hypomorphic p19^{Arf} mutants. The first deletion mutant (Δ 2–14) lacks the 13 residues downstream of the initiator methionine, is defective in binding to both Mdm2 and NPM, and is degraded more rapidly than WT p19^{Arf} (36, 37). The second deletion mutant (Δ 26–37) is able to interact with both Mdm2 and NPM *in vitro* but is unable to enter the nucleolus efficiently, although its stability is not highly compromised (36, 37). When introduced by transient transfection into 293T cells, these two Arf mutants were defective in inducing sumoylation of Mdm2 (Fig. 3A) and NPM (data not shown). Immunofluorescence studies using retroviral vector-infected NIH 3T3 cells confirmed that Arf (Δ 26–37) was excluded from nucleoli, whereas Arf (Δ 2–14), although still accumulating there, did not efficiently localize SUMO-1-conjugated molecules to nucleoli (Fig. 3B Top), even though the different Arf proteins were expressed at nearly equivalent levels (see p19^{Arf} blot). We obtained similar results with primary MEFs lacking both Arf and p53 (Fig. 3B Bottom). Taken together, we conclude that (i) p19^{Arf} induces sumoylation of proteins with which it physically interacts, (ii) Arf induces widespread sumoylation of nucleolar proteins, and (iii) Arf-induced sumoylation does not require p53 or Mdm2.

Inhibition of Sumoylation Does Not Overtly Affect Arf-Mdm2-p53 Signaling. The avian adenovirus CELO encodes Gam1, a protein that interferes with the activity of the SUMO-1 activating enzyme, leads to the degradation of Ubc9, and inhibits the SUMO pathway in mammalian cells (38). Given the ability of p19^{Arf} to stimulate sumoylation of selected proteins with which it interacts, we reasoned that Gam1 would function “upstream” of p19^{Arf} to block its effects on protein sumoylation. Myc-tagged versions of WT Gam1 and a defective Gam1 mutant used as a

Fig. 3. Two Arf mutants do not induce sumoylation. (A) Lysates of 293T cells cotransfected with the indicated combinations of vectors encoding different Arf protein variants (see text), SUMO-1, and Mdm2 were affinity-purified on nickel beads, separated on denaturing gels, and immunoblotted with antibody to Mdm2. (B) Cells infected with vectors encoding SUMO-1 and a control vector (empty), WT Arf, Arf $\Delta 2-14$, or Arf $\Delta 26-37$ (indicated at the top) were immunostained with antibodies to SUMO-1 or p19^{Arf} (indicated at the left). Similar levels of WT and mutant Arf proteins were expressed as documented by a representative immunoblot.

control were expressed in 293T cells (Fig. 4A). Expression of Gam1 in the presence or absence of plasmids encoding His₆-SUMO1, p19^{Arf}, and Mdm2 demonstrated that Gam1, but not the defective Gam1 mutant, blocked sumoylation of Mdm2 (Fig. 4A Upper). Sumoylation of the endogenous NPM protein also was triggered by p19^{Arf}, and again, Gam1, but not the Gam1 mutant, inhibited NPM sumoylation (Fig. 4A Lower). Only WT Gam1 reduced Ubc9 expression, even though it and the mutant form were expressed at equivalent levels (data not shown and Fig. 4C).

Because Mdm2 is sumoylated in an Arf-dependent manner, these findings raised the question of whether Mdm2 sumoylation would alter the p53 transcriptional response in any significant way. To test this, we used retroviral vectors to introduce Gam1 or the defective Gam1 mutant into MT-Arf cells together with His₆-SUMO-1 and then induced Arf 3 days later. Although Gam1, but not the Gam1 mutant, blocked Mdm2 sumoylation, p19^{Arf}, p53, Mdm2, and p21^{Cip1} were induced to equivalent levels whether Gam1 was expressed or not (Fig. 4B). Gam1 and the defective Gam1 mutant were expressed at comparable levels (Fig. 4B and C), but only WT Gam1 reduced the levels of Ubc9 (Fig. 4C). As an independent approach, we used short hairpin RNA to limit Ubc9 expression and Mdm2 sumoylation, and again observed no effects on the ability of p19^{Arf} to trigger the expression of p53-responsive genes (Fig. 4D). Hence, Arf-induced sumoylation seems not to be a major regulator of this aspect of the p53 transcriptional program.

Fig. 4. Reversal of Arf-induced sumoylation. (A) 293T cells were cotransfected with the combinations of expression vectors indicated at the top. Proteins adsorbed to nickel resin in 8 M urea were separated on denaturing gels and blotted with antibodies to Mdm2 (Upper) and NPM (Lower). (B) MT-Arf cells infected with a retroviral vector encoding His₆-SUMO-1 together with an empty control retroviral vector (CTL), or those encoding either Gam1 or an inactive Gam1 mutant, were induced with zinc sulfate for the indicated times (top). Proteins purified on nickel beads were separated and blotted with antibodies to Mdm2 to reveal sumoylation. Total unfractionated proteins from the same cell lysates were blotted with antibodies to the proteins indicated at the left. (C) Cellular proteins harvested 24 h after zinc induction were separated on separate gels and immunoblotted with antibodies to Myc-tagged Gam1 and Ubc9. (D) NIH 3T3 and MT-Arf cells infected for 48 h with a retroviral vector encoding short hairpin RNA directed to Ubc9 or with an empty control vector (CTL) were induced with zinc sulfate for 16 h, and electrophoretically separated proteins (indicated on the left) were analyzed by immunoblotting as in B and C.

Discussion

Enforced expression of p19^{Arf} can arrest proliferation of p53-null (6) and p53/Mdm2-null cells (8). Conversely, Arf inactivation in these cell types further accelerates the rate of their proliferation in culture and predisposes “triple knock-out” mice lacking Arf, Mdm2, and p53 to much more rapid and extensive tumorigenesis than that in mice lacking Mdm2 and p53 (8). The demonstration that human and mouse Arf proteins induce sumoylation provides a tantalizing clue as to how p19^{Arf} may mediate biological effects in the absence of Mdm2 and p53. Both human p14^{ARF} and mouse p19^{Arf} promote accumulation of SUMO-1-conjugated (H)Mdm2 (19, 20), and sequences in the Arf proteins that are required for their direct and efficient physical association with Mdm2 (residues 2–14 in p19^{Arf}) also are necessary for sumoylation. The same residues mediate efficient binding to the abundant nucleolar protein NPM (37, 40), which also was not sumoylated in response to Arf Δ2–14. Arf induction did not trigger sumoylation of several other proteins to which p19^{Arf} does not strongly bind, arguing for additional specificity. Overexpression of Arf can change the subcellular localization of Mdm2 by promoting its import into the nucleolus where most p19^{Arf} protein accumulates (36). In MT-Arf cells, the accumulation of induced p19^{Arf} in nucleoli resulted in an extensive redistribution of sumoylated molecules from the nucleoplasm to nucleoli. An Arf mutant lacking residues 26–37, which does not efficiently enter nucleoli but still binds to Mdm2 with high affinity, also was defective in Mdm2 sumoylation. Taken together, these results argue that Arf-induced sumoylation depends on a direct physical interaction of p19^{Arf} with either Mdm2 or NPM, and that this process may preferentially occur in the nucleolus where the major pool of p19^{Arf} resides.

Expression of Arf proteins protects p53 from Mdm2-mediated ubiquitination, leading to p53 stabilization and induction of p53-responsive genes. Introduction of the adenoviral Gam1 protein or of short hairpin RNA to Ubc9 into cells blocked the SUMO pathway and inhibited Arf-induced sumoylation of Mdm2 without affecting p53 stabilization or the induction of both Mdm2 and p21^{Cip1}. Thus, sumoylation appears not to be required for activation of the p53 transcriptional program per se, although more subtle effects on gene expression cannot be precluded. Enforced expression of p19^{Arf} in cells lacking functional p53 increased the sumoylation of endogenous target proteins such as NPM. In turn, Gam1-mediated inhibition of NPM sumoylation had no effect on Arf's ability to retard rRNA processing (M. Sugimoto, K.T., and C.J.S., unpublished data). Hence, putative biological effects of Arf-induced sumoylation must lie elsewhere. Because overexpression of Gam1 or interference with Ubc9 expression further inhibits cell-cycle progression and is ultimately toxic to cells, we were unable to determine whether p19^{Arf}-mediated sumoylation contributes in any way to Arf's effects on proliferation.

Human p14^{ARF} has been reported to bind to several other proteins, including the WRN helicase, E2F1, c-Myc, topoisomerase I, HIF-1α, the adenovirus E1A-regulated transcriptional repressor p120^{E4F}, and the HIV Tat-binding protein 1 (12, 13, 16, 17, 21, 22, 41). With the exception of c-Myc, which has not been tested, these proteins can undergo sumoylation when cotransfected with p14^{ARF} and His₆-SUMO-1 into human tumor cell lines. Like ARF, transfected WRN predominantly localized to nucleoli, but unlike the observed increase in overall nucleolar SUMO-1 immunofluorescence signals observed after p19^{Arf} induction in our studies, p14^{ARF} overexpression redistributed the ectopically expressed WRN protein from nucleoli to other sites within the nucleus (21). This result suggests that p14^{ARF}-induced sumoylation might regulate nucleolar export, rather than import, of certain modified target proteins, although an important

caveat is that the achieved levels of p14^{ARF} and WRN expression eclipsed those of normal cells.

Is Arf a component of a SUMO E3 ligase? For example, might p19^{Arf} serve as a specificity factor that bridges Ubc9-containing complexes to Arf-binding proteins? Woods *et al.* (21) stated that robust SUMO-1 conjugation of WRN could be observed in an *in vitro* reconstitution reaction in the presence of the SUMO E1/E2 enzymes but indicated that addition of recombinant p14^{ARF} had no effect, even at limiting concentrations of Ubc9 with which tagged p14^{ARF} can coprecipitate when overexpressed (22). An inability to stimulate sumoylation *in vitro* with recombinant p14^{ARF} may not be surprising given the fact that Arf proteins are highly basic, bind nonspecifically to many acidic proteins and to nucleic acids, and normally reside in “buffered” higher-molecular-mass complexes in living cells (40). Hence, a better approach might be to test native Arf protein complexes purified from mammalian cells for an associated E3 ligase activity when combined *in vitro* with the other purified components of the SUMO cascade. Because Gam1 interferes with SUMO E1 activity and lowers Ubc9 levels, p19^{Arf} must function “downstream.” If p19^{Arf} is a selectivity component of a SUMO E3 ligase, it should bind both to Ubc9 and to its protein targets. Thus, p19^{Arf} would have no effect in modifying other nonassociating proteins such as RanGAP1 or HDACs, which are bona fide SUMO substrates but were unaffected by p19^{Arf} in our studies. Such a model would also demand that any direct binding of p19^{Arf} to target proteins such as Mdm2 or NPM on the one hand and to Ubc9 on the other would have to be mediated through separate binding motifs within Arf, a prediction that has not so far been rigorously tested. A conceptual complication is that the domains of mouse p19^{Arf} that are required for Mdm2 binding reside completely within sequences encoded by exon 1 (36), whereas those in human p14^{ARF} are encoded by segments within exons 1 and 2 (42). Alternatively, Arf-induced sumoylation might be mediated through an ability to inhibit a deconjugating SUMO protease. However, transfection of one such enzyme, SENP1, into cells prevented the sumoylation of Hdm2 even when cotransfected with p14^{ARF}, implying that Arf does not inhibit the protease activity of SENP1 (20).

Mammalian transcription factors that control cell proliferation are sumoylated (23–25, 32), and the effects of Gam1 on sumoylation can increase the activity of several such factors (38). Gam1 reduces the sumoylation of HDAC1 without affecting its enzymatic activity (43), but HDAC1 recruitment to promoters is enhanced when histone H4 is sumoylated (44). Sumoylation of Elk-1 recruits suppressive HDAC activity to Elk-1-regulated promoters (45), and conversely, the SENP1 protease enhances androgen receptor-dependent transcription by desumoylation of HDAC1 (46). Observations that Arf binds to and/or negatively regulates transcription factors such as E2F-1 (12, 13), Myc (16, 17), and NFκB (18) raise the question of whether Arf-mediated sumoylation may account for any of these effects. In proteome-wide surveys in *Saccharomyces cerevisiae*, many functional categories of SUMO-modified proteins were detected, including those involved in transcription and chromatin remodeling, DNA repair, ribosome synthesis, metabolism, and various stress responses (47–50). It is, therefore, conceivable that sumoylation triggered by mouse p19^{Arf} in *S. cerevisiae* might lead to phenotypes that would allow a further dissection of this process in a simple eukaryote that is highly amenable to genetic manipulation.

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