p53-Dependent and -Independent Functions of the Arf Tumor Suppressor

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The *Ink4a-Arf* locus encodes two closely wedded tumor suppressor proteins (p16[Ink4a] and p19[Arf]) that inhibit cell proliferation by activating Rb and p53, respectively. With few exceptions, the *Arf* gene is repressed during mouse embryonic development, thereby helping to limit p53 expression during organogenesis. However, in adult mice, sustained hyperproliferative signals conveyed by somatically activated oncogenes can induce *Arf* gene expression and trigger a p53 response that eliminates incipient cancer cells. Disruption of this tumor surveillance pathway predisposes to cancer, and inactivation of *INK4a-ARF* by deletion, silencing, or mutation has been frequently observed in many forms of human cancer. Although it is accepted that much of Arf's tumor-suppressive activity is mediated by p53, more recent genetic evidence has pointed to additional p53-independent functions of Arf, including its ability to inhibit gene expression by a number of other transcription factors. Surprisingly, the enforced expression of Arf in mammalian cells promotes the sumoylation of several Arf-interacting proteins, implying that Arf has an associated catalytic activity. We speculate that transcriptional down-regulation in response to Arf-induced sumoylation may account for Arf's p53-independent functions.

Mammalian INK4 proteins are specific polypeptide inhibitors of the cyclin D-dependent kinases (Cdk4 and Cdk6) whose expression helps to maintain proteins of the retinoblastoma family (RB, p107, and p130) in their active hypophosphorylated states. By doing so, the INK4 proteins enhance the growth-suppressive activities of RB and its relatives, thereby preventing cells from entering the DNA synthetic (S) phase of the cell division cycle and reinforcing RB-dependent transcriptional programs that regulate cell differentiation and senescence. The founding member of the *INK4* gene family, *INK4a* (Serrano et al. 1993), is one of four genes whose encoded products act in a biochemically similar manner to inhibit the Cdk4 and Cdk6 kinases (Roussel 1999; Ortega et al. 2002). In humans, *INK4a* and *INK4b* are closely linked on the short arm of chromosome 9, whereas the two other family members (*INK4c* and *INK4d*) reside on chromosomes 1 and 19, respectively. In the mouse, however, *Ink4a*, *Ink4b*, and *Ink4c* all map to chromosome 4 with *Ink4c* separated from the *Ink4a/Ink4b* cluster by 18 cM. The *Ink4c* and *Ink4d* genes are ubiquitously expressed in highly stereotypic patterns in the mouse embryo, and their loss of function can severely compromise the proper development of certain tissues. In contrast, neither *Ink4a* nor *Ink4b* is expressed during development or, at appreciable levels, in tissues of young mice (Zindy et al. 1997, 2003), and their inactivation in the germ line does not lead to obvious developmental anomalies (Serrano et al. 1996; Latres et al. 2000; Krimpenfort et al. 2001; Sharpless et al. 2001). Instead, their induction occurs in response to particular cellular stresses that can contribute to tumor formation (Lowe and Sherr 2003). At least three Ink4 proteins (p16[Ink4a], p15[Ink4b], and p18[Ink4c]) are tumor suppressors, but by far, the most frequently inactivated family member in human cancers is *INK4a* (Ruas and Peters 1998).

Remarkably, the mouse *Ink4a* locus was found to encode yet another tumor suppressor protein, p19[Arf] (p14[ARF] in humans) whose expression induces p53 (Quelle et al. 1995; Kamijo et al. 1997). The organization of the locus—now designated *Ink4a-Arf*—is unusual in the sense that the two protein products are encoded in part by alternative reading frames (from which *Arf* takes its name) within exon 2 (Fig. 1A). Because the *Ink4a* and *Arf* genes have alternative first exons and separate promoter elements, they can be independently regulated, mutated, and epigenetically silenced. Together, *INK4a*, *ARF*, *RB*, and *TP53* represent the most frequently inactivated tumor suppressors in human tumors, suggesting that their loss of function might be a necessary prerequisite for the transformation of normal cells into cancer cells. Here, we review *Arf*'s activities and discuss insights stemming from recent studies.

p19[Arf] INDUCES p53—THE ORIGINAL PARADIGM

Enforced expression of the p19[Arf] protein arrests cells in both the G_1 and G_2 phases of the cell division cycle (Quelle et al. 1995). Much of the growth-suppressive function of p19[Arf] depends on its ability to interfere with the p53 negative regulator Mdm2 (HDM2 in humans), which itself is a p53-responsive gene. Arf stabilizes p53 by blocking Mdm2's activity as an E3 ubiquitin protein ligase (Honda and Yasuda 1999). It can also relocalize Mdm2 into nucleoli, where the bulk of intracellular p19[Arf] resides (Weber et al. 1999), although this may not be necessary for p53 induction (Llanos et al. 2001). Whatever the exact mechanisms involved, growth arrest by p19[Arf] depends to a great extent on p53-dependent transcription, which up-regulates many antiproliferative
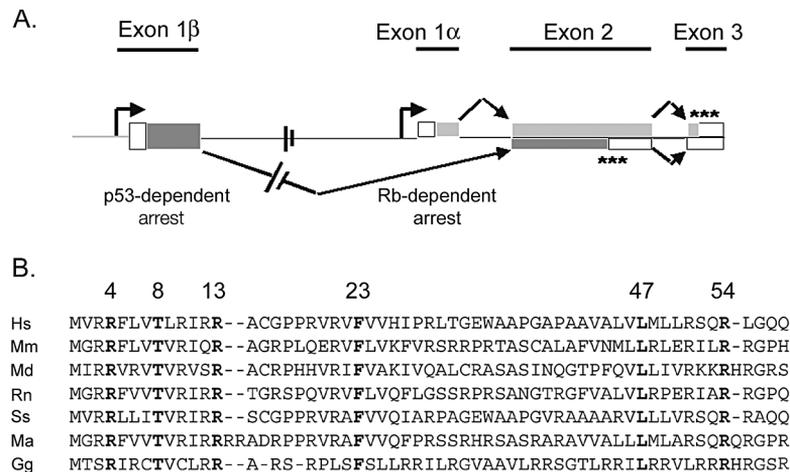
A.

Figure 1. Organization of the *Ink4a-Arf* locus and comparison of amino acid sequences encoded by *Arf* exon 1β in various species. (*A*) The *Ink4a* gene is composed of three exons (1α, 2, and 3 indicated by boxes). Coding segments are indicated by light gray shading above the horizontal line whereas noncoding segments are unshaded. The *Arf* gene is represented by exons 1β (~15 kb upstream), 2, and 3. Coding sequences (*dark gray shading*) in exon 2 are in an alternative reading frame from that which encodes p16^{Ink4a}. Arrows indicate the two promoters and splicing patterns. Asterisks denote the positions of termination codons. Strains of knockout (KO) mice include animals that lack both gene products (Serrano et al. 1996), *Arf* alone (Kamijo et al. 1997), and *Ink4a* alone (Krimpenfort et al. 2001; Sharpless et al. 2001). Sequences encoding GFP have also been knocked into (KI) the Arf locus replacing exon 1β (Zindy et al. 2003). (*B*). Amino-terminal sequences encoded by *Arf* exon 1(from *Homo sapiens* (Hs), *Mus musculus* (Mm), *Monodelphis domestica* (opossum) (Md), *Ratus norvegicus* (Rn), *Sus scrofa* (domestic pig) (Ss), *Mesocricetus auratus* (golden hamster) (Ma), and *Gallus gallus* (chicken) (Gg). The positions of six universally conserved residues are denoted by bold type and by numbers above the figure. (Adapted, with permission, from Sherr [1998] and Kuo et al. [2004].)

genes, such as the Cdk inhibitor p21^{Cip1} (El-Deiry et al. 1993), as well as a subset of genes that contribute to p53's pro-apoptotic functions (Vousden and Lu 2002). However, p19^{Arf} can arrest the division of cells lacking *Cip1* (Pantoja and Serrano 1999; Modestou et al. 2001) as well as those lacking *p53* alone or both *p53* and *Mdm2*, albeit much less efficiently than in cells that preserve p53 function (Carnero et al. 2000; Weber et al. 2000b). Thus, *Arf* has both p53-dependent and -independent activities.

Animals lacking *Arf* are highly tumor-prone, although spontaneous tumors occur with a rate and spectrum that differ from those of animals lacking *p53* (Jacks et al. 1994; Kamijo et al. 1999a). Whereas *p53*-null mice tend to develop lymphomas and die within 6 months of birth, the *Arf*-null animals live much longer (up to 15 months) and have a propensity to develop more sarcomas than lymphomas. In addition, about 25% of *Arf*-null animals develop gliomas and carcinomas, which rarely arise spontaneously in mice lacking *p53*. Although *Arf* is activated in response to various forms of stress (see below), it is not acutely induced in response to DNA double-strand breaks triggered by ionizing irradiation, although *Arf*'s absence can enhance Mdm2's ability to down-regulate p53 during the DNA damage response (Kamijo et al. 1997, 1999b). Triple knockout (TKO) mice lacking *Arf*, *Mdm2*, and *p53* rapidly develop many more tumors—and frequently, multiple tumors of different histological types per animal—than mice lacking both *Mdm2* and *p53* (Weber et al. 2000b). Indeed, most TKO animals die of various cancers before reaching breeding age. Although obtained with difficulty, TKO mouse embryonic fibroblasts (MEFs) can be growth-arrested when p19^{Arf} expression is reinforced. Together, these results underscore the ability of p19^{Arf} to interact with targets other than Mdm2 and p53.

Arf REGULATES REPLICATIVE SENESCENCE

Although *Arf* is expressed in the yolk sac and embryonic eye (see below), it is not appreciably expressed in other tissues within the mouse embryo, implying that the Arf–Mdm2–p53 pathway is generally shut off during development in utero (Zindy et al. 1997, 2003). However, explantation and passage of primary MEFs in tissue culture leads to *Arf* induction with a steady increase in its protein levels that correlates with the waning proliferative capacity of continuously propagated cells (Kamijo et al. 1997; Zindy et al. 1998). This seemingly spontaneous activation of p19^{Arf} in cultured MEFs results in their replicative senescence. Conversely, MEFs explanted from *Arf*-null animals not only proliferate faster than their wild-type counterparts in culture, but also do not senesce and behave like established mouse cell lines (Kamijo et al. 1997). Moreover, immortalized cell populations that arise stochastically as wild-type MEFs are continuously propagated in culture either acquire *p53* mutations (Harvey and Levine 1991) or sustain bi-allelic *Ink4a-Arf* deletions (Kamijo et al. 1997). These findings pointed to the idea that *Ink4a* and *Arf* are induced in response to the nonphysiological tissue culture milieu ("culture shock") (Sherr and DePinho 2001). The rate of *Arf* induction can be accelerated by introducing oncogenes such as activated Myc, Ras, or adenovirus E1A into primary cell strains (Zindy et al. 1998; De Stanchina et al. 1998; Palmero et al. 1998) or by subjecting MEFs to low but persistent levels of oxidative stress (Busuttil et al. 2003; Parrinello et al. 2003). *Ink4a* is also induced by many of the same stress stimuli that impinge upon *Arf*, but even though p16^{Ink4a} levels continue to rise to a maximum during continuous passage of MEFs in

culture, *Ink4a* loss alone in MEFs does not bypass senescence (Krimpenfort et al. 2001; Sharpless et al. 2001). Still, the determinants of the senescence response of mouse cells are to some extent cell-type-specific. For example, bone-marrow-derived pre-B cells can be immortalized through *Arf* inactivation, whereas immortalization of bone-marrow-derived macrophages explanted from *Arf*-null animals depends on the inactivation of *Ink4a*, which undergoes epigenetic silencing (Randle et al. 2001).

The co-inactivation of *INK4a* and *ARF* (or co-elimination of both *RB* and *TP53*) endows human cells with an increased cellular life span in culture. However, these events are not sufficient to immortalize human cells in which replicative senescence is also triggered by telomere malfunction (Wright and Shay 2000). MEFs have considerably longer telomeres than those of human fibroblast strains, and they have a greater propensity to express telomerase and maintain telomere length. Engineered acute disruption of mouse telomere integrity activates the *Arf* checkpoint, whereas both *ARF* and *INK4a* respond in human cells (Smogorzewska and De Lange 2002). On the basis of these and other considerations, it has been suggested that *INK4a* may play a more significant role in governing the senescence of human versus mouse cells (Wright and Shay 2000). By genetically eliminating telomerase activity in the mouse germ line and interbreeding the progeny, later generations of telomerase-null mice that developed shorter telomeres were forced to confront the problem of telomere insufficiency (Blasco et al. 1997). Cells from these "humanized" mouse strains not only activated Rb- and p53-dependent checkpoints in response to telomere shortening but, in the absence of these checkpoints, manifested progressive telomere attrition and eventually entered "crisis" (Lee et al. 1998; Rudolph et al. 1999). The latter event is characterized by end-to-end chromosome fusions and fusion-bridge-breakage cycles that destabilize the genome and trigger mitotic catastrophe. Because cells from these mice cannot reactivate telomerase activity, they can only restabilize their telomeres through alternative recombination-based mechanisms. Mice lacking telomerase and intact checkpoint controls spontaneously develop a telling spectrum of tumors, the majority of which are carcinomas that are rarely observed in wild-type mice (Artandi et al. 2000). This response to checkpoint disruption and telomere attrition closely reflects the human cancer condition in which carcinomas predominate as we age.

ACTIVATING *Arf* GENE EXPRESSION

Abnormal hyperproliferative signals conveyed by oncogenes can remodel the otherwise insulated *Arf* promoter to enable its expression (Lowe and Sherr 2003). In turn, the ability of p19^{Arf} to activate p53 provides a safeguard that protects such cells from oncogenic challenge. In a Burkitt's lymphoma model in which a *Myc* transgene is driven by an immunoglobulin promoter-enhancer (Eµ-*Myc* mice) (Adams et al. 1985), *Arf* induction in *Myc*-expressing B cells limits Myc's pro-proliferative effects early in the course of disease. However, continued Myc

expression eventually selects for the appearance of cells that have sustained *p53* mutations or *Arf* deletions, thereby allowing lymphomas to arise (Eischen et al. 1999; Jacobs et al. 1999b; Schmitt et al. 1999). Eµ-*Myc*-induced lymphomagenesis is greatly accelerated in an *Arf*$^{+/-}$ genetic background where the wild-type *Arf* allele is rapidly inactivated; in an *Arf*$^{-/-}$ background, animals become moribund very soon after birth, and all die within 8 weeks of highly aggressive lympholeukemias with widespread visceral involvement (Eischen et al. 1999). Similar effects of *Arf* on tumor suppression have been observed in carcinogen-induced skin tumors where somatic mutation of Ras is the initiating step (Kelly-Spratt et al. 2004). Importantly, Myc or Ras signaling in normal cells, although important for their proliferative response to mitogens, is insufficient to activate *Arf*. Therefore, *Arf* acts as a fuse to monitor mitogenic "current" and to trigger a p53 response only when mitogenic signals are abnormally sustained and/or elevated above a potentially deleterious threshold level.

Strikingly, after replacement of Arf exon 1β genomic sequences with those encoding green fluorescent protein (GFP), tumors arising in functionally *Arf*-null *Arf*$^{GFP/GFP}$ homozygous mice were vividly fluorescent, whereas the non-tumor tissues in moribund mice did not express GFP (Fig. 2A,B,D) (Zindy et al. 2003). When the Eµ-*Myc* transgene was expressed in *Arf*$^{GFP/+}$ heterozygotes, the wild-type allele was lost and lymphomagenesis was accelerated relative to the rate of tumor formation in *Arf*$^{+/+}$
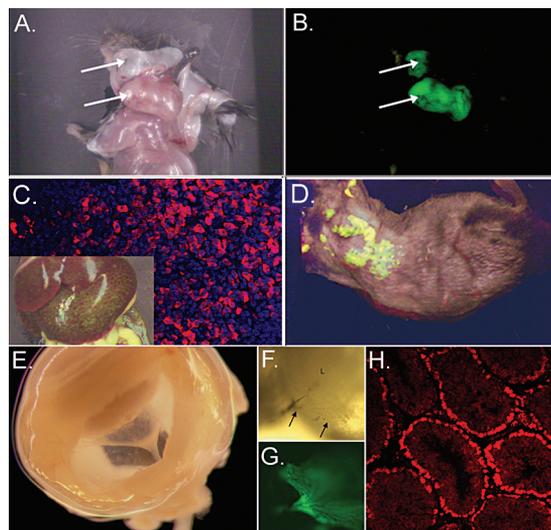

Figure 2. GFP expression in tissues of *Arf* GFP/GFP mice. (*A,B*) A green fluorescent fibrosarcoma in the neck region. (*C*) Macroscopic foci of GFP-positive lymphoma cells in the liver and a microscopic metastasis (*inset*) visualized by immunofluorescence (*red*) and counterstained with 4′,6-diamidino-2-phenylindole (*blue*). (*D*) Whole-body imaging of a shaved Arf$^{+/GFP}$ Eµ-*Myc* transgenic mouse with lymphoma. (*E*) Whole mount showing a funnel-shaped mass stretching from the lens (*top left*) toward the optic cup (*rear*). A closer view shows elements of the hyaloid vasculature (*F, arrows*) within the fluorescent mass (*G*). (*H*) Immunohistochemical staining of GFP (*red*) in the testis of an 8-month-old mouse. (Reprinted, with permission, from Zindy et al. 2003.)

littermates. Early in the course of disease, we detected GFP-positive cells in the bone marrow, spleen, and lymph nodes of Eμ-*Myc* transgenic *Arf*^{GFP/+} mice, implying that *Arf* induction could be used to monitor precancerous lesions. In turn, once lymphomas arose, their metastatic spread was marked by the appearance of vividly fluorescent tumor nodules in various organs (Fig. 2C). Using a monoclonal antibody to p19^{Arf} that detects the endogenous protein in fixed lymphoid cells (Bertwistle et al. 2004b), we hope to determine how early in the course of Eμ-Myc-induced disease the endogenous Arf protein is induced.

Interestingly, with few exceptions, very few cells in young, normal *Arf* $^{GFP/GFP}$ homozygotes exhibited green fluorescence. However, *Arf*-null mice become blind soon after birth due to the formation of a cellular mass in the vitreous that disrupts the normal architecture of the lens and retina. *Arf* expression in the vitreous during the first week after birth is required for regression of the hyaloid vasculature system that nourishes the developing lens (McKeller et al. 2002). In newborn *Arf*-null mice, persistence of the hyaloid vasculature leads to an abnormal proliferation of perivascular cells that form a funnel-shaped mass in the vitreous (Fig. 2E,F). In *Arf* $^{GFP/GFP}$ animals, the retrolental mass included green fluorescent cells (Fig. 2G). *Arf* expression could also be observed in testicular tubules, in cells that most likely represent spermatogonia or leptotene spermatocytes at the earliest stages of meiosis I (Fig. 2H). Small pockets of green fluorescent cells could be seen in other ostensibly normal tissues, including the thymic medulla and lung alveoli, although such cells were rare and expressed only low levels of GFP detected by immunohistochemistry (Zindy et al. 2003).

Understanding how the *Arf* promoter distinguishes normal and abnormal signaling thresholds remains problematic. *Arf* is normally repressed by E2F complexes (Rowland et al. 2002), but unlike many E2F-responsive genes that govern DNA synthesis and replication, *Arf* is not periodically expressed when cells enter S phase. Yet, when *Arf* is induced by oncogenes, E2Fs 1, 2, and 3a replace repressive E2F complexes on the *Arf* promoter (Aslanian et al. 2004). Hence, other specificity factors must play a role in modulating this E2F response. An attractive candidate is the Dmp1 transcription factor, which binds adjacent to an E2F site in the proximal *Arf* promoter to activate the gene (Inoue et al. 1999). Several features suggest that Dmp1 plays a role in regulating *Arf* induction mediated by oncogenes: (1) *Dmp1* is a Ras-responsive gene that is activated via the Raf–MEK–ERK pathway (Sreeramaneni et al. 2005); (2) *Dmp1* overexpression in response to oncogenic Ras induces cell cycle arrest in primary MEFs in an *Arf*-dependent manner (Inoue et al. 1999); (3) conversely, *Dmp1*-null MEFs bypass senescence with relative ease and can be transformed even at early passage by oncogenic Ras at a detectable frequency (Inoue et al. 2000); (4) *Dmp1*$^{–/–}$ and *Dmp1*$^{+/–}$ mice, like *Arf*-null animals, are highly sensitive to Eμ-*Myc*- or carcinogen-induced lymphomagenesis, and in these settings, *Dmp1* is haplo-insufficient for tumor suppression; (5) inactivation of *Dmp1* reduces the frequency of *Arf* loss and *p53* mutation during Eμ-*Myc*-induced lymphomagenesis, indicating that *Dmp1*

is a genetic modifier of the Arf–Mdm2–p53 signaling pathway (Inoue et al. 2001). It will be of interest to determine the status of various E2F complexes on the *Arf* promoter in cells lacking *Dmp1* and in those reconstituted with wild-type and mutant *Dmp1* alleles.

Arf is subject to repression by a variety of other proteins, including Bmi1, Tbx2 and Tbx3, Jun D, Twist, CBX7, and Pokemon (Jacobs et al. 1999b, 2000; Maestro et al. 1999; Weitzman et al. 2000; Lingbeek et al. 2002; Gil et al. 2004; Maeda et al. 2005). Some of these repressors are likely to be responsible for silencing the *Arf* locus during development. In particular, inactivation of *Bmi1* results in inappropriate *Ink4a-Arf* expression in cerebellar granule neuron precursors and in adult hematopoietic stem cells, so that *Bmi1*-null animals exhibit defects in postnatal cerebellar development and die early in life from bone marrow aplasia. These defects are rescued on an *Ink4a-Arf*-null background, indicating that abnormal activation of the locus can interfere with these developmental processes (Jacobs et al. 1999a). In contrast, multiple embryonic defects arising in mice lacking *Twist* (W. den Besten, unpubl.) or yolk sac atresia occurring in homozygous *Tbx3*-null mice (Jerome-Majewska et al., in press) are not rescued in an *Arf*-null background.

THE p19^{Arf} PROTEIN: A CHALLENGE FOR BIOCHEMISTS

Arf proteins are highly basic (pI ~12) and contain ~20% arginine but only one (p19^{Arf}) or no (p14^{ARF}) lysines. Truncation of the mouse *Arf* gene near the exon 1β-exon 2 junction yielded a polypeptide of 64 amino acids that exhibited the growth-suppressing activity of the parental full-length molecule (Quelle et al. 1997). In this regard, chickens lack the *Ink4a* locus but have an *Arf* gene specifying a 7-kD protein encoded almost exclusively by exon 1β sequences (Fig. 1B) (Kim et al. 2003). Most recombinant p19^{Arf} protein expressed in bacteria is insoluble, and many arginyl codons are improperly translated. Reasoning that the more abundant species of bacterial arginyl tRNAs do not efficiently recognize a subset of mammalian arginine codons, we used synthetic oligonucleotides to construct an *Arf* minigene encoding the amino-terminal 62 amino acids of p19^{Arf}; in doing so, many codons were altered to improve translation in bacteria (Weber et al. 2000a). Although the correct product was produced, much of it remained insoluble and worthless for "test tube biochemistry" or for structural analysis.

Given the fact that Arf proteins must function at neutral pH, they must be highly buffered within living cells. Using tandem affinity-tagged p19^{Arf}, we purified Arf-associated proteins from mouse fibroblasts undergoing cell cycle arrest and identified them by mass spectroscopy. TAP-tagged Arf associated with many proteins, the most prominent being nucleophosmin (NPM/B23), an abundant and highly acidic nucleolar protein (Bertwistle et al. 2004a). Although only a small fraction of cellular NPM binds to p19^{Arf}, most of the Arf protein in cells is associated with NPM, and this interaction occurs equally well in primary cells lacking Mdm2 and p53. When p19^{Arf} expression is induced, it colocalizes with NPM in very high molecular

weight complexes of 2–5 MD. The biological meaning of the Arf–NPM interaction remains unclear. One group of investigators suggested that p19[Arf] can impede NPM nucleocytoplasmic shuttling (Brady et al. 2004), whereas others have argued that NPM helps to sequester p19[Arf] in nucleoli (Korgaonkar et al. 2005). All agree that p19[Arf] binds NPM through the same domains that mediate its binding to Mdm2, so that the latter two proteins can compete with one another for p19[Arf] binding. When associated with NPM, p19[Arf] is highly stable and turns over with a half-life of 6–8 hours (Kuo et al. 2004). Overexpression of NPM further slows p19[Arf] turnover, whereas "knocking down" NPM with shRNA has the opposite effect. An Arf mutant lacking amino-terminal residues 2–14 or another in which alanines were substituted for all six conserved residues encoded by exon 1β (Fig. 1B) still localized to nucleoli but did not bind with high affinity to either Mdm2 or NPM and was handicapped in inducing growth arrest. These two mutant proteins turn over with half-lives of only 90–120 minutes (Kuo et al. 2004). Thus, the stoichiometric binding of p19[Arf] to nucleolar NPM might enable Arf to fold into a stable conformation, whereas unbound p19[Arf] mutants might be aberrantly folded and subject to more rapid degradation.

The absence of lysine residues in human p14[ARF] implied that its turnover did not depend on ubiquitination. However, both mouse p19[Arf] and human p14[ARF] undergo amino-terminal ubiquitination and proteasomal degradation (Kuo et al. 2004). Most eukaryotic proteins are acetylated at their amino termini and are therefore not substrates for amino-terminal ubiquitination. N-acetylation is favored by the presence of amino-terminal methionine, which can be cleaved by methionine aminopeptidase when the penultimate amino acid has a small radius of gyration (Bradshaw et al. 1998). Proteolytic exposure of certain amino-terminal residues, such as serine, allows acetylation to proceed efficiently, whereas other amino acids, such as glycine, are poor substrates (Polevoda and Sherman 2003). In addition, penultimate acidic residues promote acetylation whereas basic residues inhibit it. The mouse p19[Arf] amino-terminal tripeptide, Met-Gly-Arg, is processed by methionine aminopeptidase, and the resulting Gly-Arg dipeptide strongly blocks acetylation, allowing amino-terminal ubiquitination. Arf mutants that were engineered to contain more favorable amino-terminal consensus sequences for acetylation were more stable than wild-type p19[Arf], indicating that amino-terminal ubiquitination can regulate the rate of Arf protein turnover (Kuo et al. 2004). In high-molecular-mass complexes with NPM, p19[Arf] may require ubiquitination for its unfolding and subsequent destruction, but unstable Arf mutants, although still subject to amino-terminal ubiquitination, may be less dependent on this process for their destruction.

p53-INDEPENDENT FUNCTIONS OF Arf

Despite cogent genetic arguments that p19[Arf] has tumor suppressor activities that do not depend on Mdm2 and p53, the mechanism(s) underlying Arf's p53-independent functions remain unclear. Arf can physically or functionally interact with a variety of proteins, including the transcription factors E2F-1, Myc, NF-κB, and HIF-1α, nucleolar proteins including NPM and nucleolin, topoisomerase I, the Werners helicase (WRN), cyclin G1, the adenovirus E1A-regulated transcriptional repressor p120[E4F], and the HIV Tat-binding protein (Eymin et al. 2001; Martelli et al. 2001; Rocha et al. 2003; Zhao et al. 2003; Ayrault et al. 2004; Bertwistle et al. 2004a; Datta et al. 2004; Qi et al. 2004; Woods et al. 2004; Rizos et al. 2005). Although these interactions are of great potential interest, there are many caveats in interpreting their biological relevance. Most of these protein–protein interactions have been uncovered through antibody-mediated coprecipitation, frequently of overexpressed proteins. Because Arf is so highly basic and has a strong propensity to nonspecifically bind to nucleic acids and acidic proteins, it is difficult to devise appropriate experimental controls for such analyses. In many cases, mutagenesis has been employed to pinpoint domains within Arf and its target proteins that govern these interactions. However, a common finding has been that multiple segments within Arf and its associated proteins seem to contribute, thereby negating the possibility of identifying discrete motifs that direct specific binding. These problems are further compounded by observations that Arf mutants lacking particular segments are highly unstable or localize aberrantly when expressed in cells (see above).

Despite these difficulties, however, certain emerging themes merit strong consideration. Induction of p19[Arf] can inhibit the production of ribosomal RNA by affecting the processing of 47/45S and 32S precursors (Sugimoto et al. 2003). These effects are not strictly dependent on inhibition of rRNA transcription, do not require p53, and are not mimicked by cell cycle arrest per se. Hence, p19[Arf] may play a distinct role within the nucleolus, possibly in coordinating ribosome synthesis and cell growth (mass) with p53-dependent proliferative arrest.

Arf has also been demonstrated to inhibit gene expression through functional interactions with various transcription factors, including E2F-1 (Eymin et al. 2001; Martelli et al. 2001), NF-κB (Rocha et al. 2003), and Myc (Datta et al. 2004; Qi et al. 2004). Again, none of these effects depends on p53. There is some evidence that p19[Arf] can physically associate with E2F-1- and Myc-containing protein complexes (see above), but a direct interaction with NF-κB has not been reported. Overexpression of p19[Arf] was found to accelerate E2F-1 degradation (Martelli et al. 2001), although this might potentially be due to an increase in E2F-1 turnover stemming from Arf-induced cell cycle arrest. In contrast, Arf has no apparent effect on Myc or NF-κB protein levels.

By repressing the transcriptional activity of NF-κB, ARF sensitizes cells to TNFα-induced apoptosis (Rocha et al. 2003). In human cells responding to a conditionally regulated *ARF* transgene, induction of p14[ARF] was reported to trigger ATR- and Chk-1-dependent phosphorylation of the NF-κB RelA/p65 subunit on Thr-505, inhibiting its *trans*-activating function; ATR induction also resulted in p53 phosphorylation on Ser-15, a modification associated with p53 activation (Rocha et al. 2005). However, phosphorylation of RelA/p65, although dependent on ARF, ATR, and Chk1, was not observed when ATR

and Chk1 were activated by UV irradiation, suggesting that p14[ARF] is somehow required to target Chk1 kinase activity to specific substrates. Although ARF might conceivably activate ATR by interfering with DNA replication, ARF-expressing cells accumulate in G_1 and G_2 with very few cells remaining in S phase, and analysis of arrested cells with an antibody to γ-H2AX revealed no increase in staining. The endogenous ATR protein was relocalized, together with BRCA1, to the nucleolus of ARF-expressing cells, and p14[ARF] and ATR cofractionated in high-molecular-weight (>1 MD) complexes. In cells sustaining DNA damage, RPA binds single-stranded DNA and interacts with ATRIP to recruit ATR to stalled replication forks, but no RPA foci were observed in nucleoli following p14[ARF] induction, again yielding no direct evidence for ARF-induced DNA damage. Hence, the manner by which p14[ARF] engages the ATR–Chk1 kinase cascade remains unknown. This is a crucial issue, particularly because a recent report has indicated that various oncogenes that, on the one hand, can induce *ARF* in precancerous lesions also induce DNA damage and activate the ATR/ATM and Chk kinases via ARF-independent signaling pathways (Bartkova et al. 2005).

Myc overexpression induces *Arf* (and can also damage DNA; Vafa et al. 2002), but, in turn, p19[Arf] can inhibit Myc function (Datta et al. 2004; Qi et al. 2004). One group of investigators reported that Myc binds directly to p19[Arf], relocalizing it from the nucleolus into the nucleoplasm (Qi et al. 2004). However, in other cell lines, p19[Arf] appeared to localize Myc to the nucleolus (Datta et al. 2004). This is reminiscent of the Mdm2–Arf interaction in which overexpression of Arf localized Mdm2 to the nucleolus (Weber et al. 1999), whereas the opposite occurred when Mdm2 expression was enforced at high levels (Zhang and Xiong 1999). Many genes are activated by Myc, and these include not only those transcribed by polymerase II, but also the pol-I-responsive rDNA clusters as well as the pol-III-driven genes encoding 5S RNA and tRNAs (Gomez-Roman et al. 2003; Orian et al. 2003; Grandori et al. 2005). Thus, Myc can be active within both the nucleoplasm and the nucleolus, and its relocalization between the two compartments in response to p19[Arf] may not simply reflect an artifact of protein overexpression. A very interesting finding is that p19[Arf] can associate with Myc–Max complexes, antagonizing their *trans*-activating functions on certain promoters without impairing their transrepression of others (Qi et al. 2004). Myc binding to these promoters occurs whether or not p19[Arf] (or p53) is present, so p19[Arf] plays no role in recruiting Myc to chromatin but instead inhibits Myc's activator function in situ. Again, the underlying mechanisms remain unclear.

Arf-INDUCED SUMOYLATION

Surprisingly, the enforced expression of p14[ARF] in human cells has been observed to promote the sumoylation of several cotransfected proteins, including Hdm2, the Werners helicase, E2F-1, and HIF-1α (Xirodimas et al. 2002; Chen and Chen 2003; Woods et al. 2004; Rizos et

al. 2005). Covalent addition of the small ubiquitin-like modifiers (SUMO 1, 2, and 3) to target proteins can generate diverse effects on protein transport, ubiquitination, DNA repair and chromatid adhesion, and on gene expression (usually, but not always, down-regulation) (Melchior 2000; Johnson 2004). Therefore, we speculate that the p53-independent activities of Arf might conceivably be due to this process.

Conjugation of SUMO to lysyl ε-amino groups in proteins occurs in a manner similar to ubiquitination (Johnson 2004). First, SUMO precursors must be cleaved by a protease to yield a carboxy-terminal diglycine motif. Processed SUMO is activated in an ATP-dependent manner by an E1 enzyme and transferred to a unique E2 conjugating enzyme (Ubc9), which can then affix SUMO to target proteins. The latter process can be accelerated by one of several E3 ligases that include the nucleoporin RanBP2 and a family of PIAS proteins (protein inhibitors of activated STATs) (Johnson and Gupta 2001; Sachdev et al. 2001; Pichler et al. 2002). SUMO-specific proteases assume a dual role in the cascade, being required for initial SUMO processing as well as the removal of SUMO from protein substrates (Hay 2001).

Sequences required for the efficient binding of p14[ARF] or p19[Arf] to (H)Mdm2, although differing between the human and mouse Arf proteins, are also necessary for sumoylation (Xirodimas et al. 2002; Tago et al. 2005). Endogenous NPM, to which Arf also binds, undergoes sumoylation in response to p19[Arf] induction, but Arf did not trigger sumoylation of other known SUMO substrates with which it did not physically interact (Fig. 3) (Tago et al. 2005). The induction and accumulation of p19[Arf] leads to increased sumoylation within nucleoli; however, an
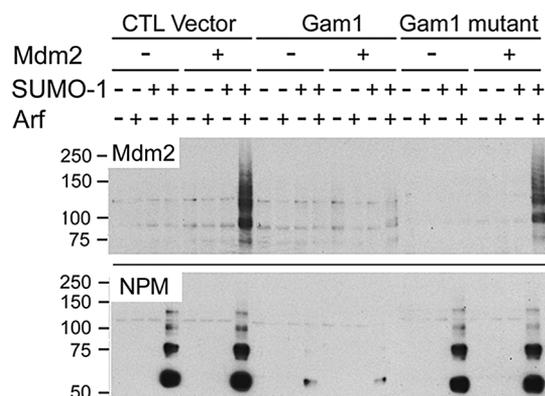

Figure 3. *Arf* induces sumoylation of Mdm2 and NPM. 293T cells were cotransfected with the combinations of expression vectors indicated at the top, which encoded Mdm2, His$_6$-tagged SUMO-1, p19[Arf], and either a control retroviral expression vector or vectors encoding wild-type Gam1 or a defective Gam1 mutant. Cellular proteins absorbed to nickel resin in the presence of 0.5 M NaCl and 8 M urea were eluted with imidazole and sodium dodecyl sulfate, electrophoretically separated on denaturing gels, and blotted with antibodies to Mdm2 (*upper panel*) or NPM (*lower panel*). The fastest-migrating species in the lower panel corresponds to the monosumoylated endogenous NPM protein, whereas slower-migrating forms are multisumoylated. (Reprinted, with permission, from Tago et al. 2005.)

Arf mutant that cannot efficiently enter nucleoli but still binds Mdm2 was defective in sumoylating it. Hence, Arf-induced sumoylation appears to depend on at least a transient association of p19^{Arf} with its targets, and this may preferentially occur within the nucleolus where the major pool of p19^{Arf} resides.

An avian adenoviral protein, Gam1, has the unique property of interfering with the SUMO E1 activating enzyme, a process that is accompanied by increased turnover of the SUMO E2, Ubc9 (Boggio et al. 2004). Either the introduction of Gam1 or shRNAs directed to Ubc9 can inhibit sumoylation within cells (Fig. 3). Although these manipulations ultimately prove toxic, inhibition of Arf-induced sumoylation of Mdm2 had no immediate effect on p53 stabilization or on the induction of canonical p53-responsive genes, such as *Cip1* or *Mdm2* itself (Tago et al. 2005). Therefore, sumoylation appears not to be needed for activation of the p53 transcriptional program per se, although we cannot preclude more subtle effects.

An attractive idea is that Arf is a component of a SUMO E3 ligase and acts as a specificity factor that bridges Ubc9 complexes to Arf-binding proteins. SUMO-1 conjugation of the WRN helicase triggered by the SUMO E1 and E2 enzymes in vitro could not be accelerated by recombinant p14^{ARF} (Woods et al. 2004). This may not be surprising, given the "sticky" binding properties of recombinant Arf proteins and their propensity to misfold, so a better approach might be to purify native Arf-containing complexes from cells and test them for an associated E3 ligase activity when recombined with potential substrates and other purified components of the SUMO cascade.

Mammalian transcription factors that control cell proliferation are sumoylated (Hilgarth et al. 2004; Johnson 2004) and Gam1 can increase promoter activation by several of them (Boggio et al. 2004). Gam1 reduces sumoylation of HDAC1 without affecting its enzymatic activity (Colombo et al. 2002), but its recruitment to promoters is enhanced when histone H4 is sumoylated (Shiio and Eisenman 2003). The fact that Arf can negatively regulate the transcriptional activities of E2F-1, NF-κB, and Myc raises the question of whether sumoylation mediates these effects. Because the key elements of the SUMO cascade are highly conserved among all eukaryotes, it is reasonable to expect that Arf might induce sumoylation-dependent phenotypes in other organisms that are more amenable to genetic dissection of this process.

CONCLUSIONS

From the time of its discovery, the properties of the Arf tumor suppressor have been surprising. The overlap of *Arf* and *Ink4a* gene sequences; the fact that the two gene products are both potent tumor suppressors that interact with p53 and Rb, respectively; the notion that Arf responds to a select subset of oncogenic signals that induce p53; the highly basic nature of Arf proteins and their localization to nucleoli; the amino-terminal ubiquitination of Arf; and recent results implicating Arf in the process of sumoylation were all unexpected. Illuminating the vari-

ous activities of Arf, and particularly its p53-independent functions, still poses a significant challenge.

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