Ubiquitination of, and Sumoylation by, the Arf Tumor Suppressor

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Key words: I*nk4a*-A*rf* locus, N-terminal ubiquitination, Arf-induced sumoylation, nucleophosmin (NPM/B23), tumor suppression, cancer

Abstract

The *Ink4a-Arf* locus, which encodes two distinct tumor suppressor proteins, is inactivated in many cancers. Whereas $p16^{Ink4a}$ is an inhibitor of cyclin D-dependent kinases, $p19^{Arf}$ ($p14^{ARF}$ in humans) antagonizes the E3 ubiquitin protein ligase activity of Mdm2 to activate p53. We now recognize that Arf functions in both p53-dependent and -independent modes to counteract hyper-proliferative signals originating from proto-oncogene activation, but its p53-independent activities remain poorly understood. Arf proteins are highly basic (> 20% arginine content, pI > 12) and predominantly localize within nucleoli in physical association with an abundant acidic protein, nucleophosmin (NPM/B23). When bound to NPM, Arf proteins are relatively stable with half-lives of 6–8 hours. Although mouse $p19^{Arf}$ contains only a single lysine residue and human $p14^{ARF}$ has none, both proteins are N-terminally ubiquitinated and degraded in proteasomes. Through as yet uncharacterized mechanisms, $p19^{Arf}$ induces p53-independent sumoylation of a variety of cellular target proteins with which it interacts, including both Mdm2 and NPM. A naturally occurring NPM mutant (NPMc) expressed in myeloid leukemia cells redirects both wild-type NPM and $p19^{Arf}$ to the cytoplasm, inhibits Arf-induced sumoylation, and attenuates p53 activity. Thus, ubiquitination and sumoylation can each influence Arf tumor suppressor activity.

IMAJ 2006;8:249–251

The mouse I*nk4a*/A*rf* genomic locus is comprised of two alternative first exons (designated 1α and 1β, respectively) whose distinct transcripts are spliced to a common mRNA segment encoded by two shared downstream exons (2 and 3). The two mRNAs are translated in alternative reading frames, so that their encoded proteins, $p16^{Ink4a}$ and $p19^{Arf}$ ($p14^{ARF}$ in humans), bear no sequence homology to one another [1]. Whereas $p16^{Ink4a}$ exerts its growth suppressive functions by activating the retinoblastoma family of proteins (pRb, p107, and p130), $p19^{Arf}$ triggers a p53-dependent transcriptional program that induces either cell cycle arrest or apoptosis, depending on the biologic setting [2]. Mutations compromising the potent tumor suppressive activities of the I*nk4a*/A*rf* locus, RB, and *p53* are hallmarks of many, if not all, cancers [3,4].

The basis of Arf tumor suppression

Although the growth-suppressive functions of $p19^{Arf}$ were initially thought to strictly depend on p53, several lines of evidence now suggest that Arf also exhibits p53-independent activities. Enforced expression of $p19^{Arf}$ in *p53*-null cells can arrest their proliferation, albeit less efficiently than in cells that retain wild-type *p53* [5,6]. Additionally, genetically engineered mice that lack *p53*, M*dm2* and A*rf* are more prone to tumor development than animals in which only M*dm2* and *p53* are co-inactivated, indicating that $p19^{Arf}$ can interact with factors other than Mdm2 and p53 to enforce tumor surveillance [6]. Numerous Arf-interacting proteins have been identified through genetic and biochemical screens; however, the functional consequences of many of these associations remain unclear. The major stumbling block has been that the highly basic nature of Arf proteins enables them to bind non-specifically to acidic molecules, making it difficult to determine the physiologic relevance of these interactions, particularly under circumstances in which protein expression has been artificially enforced.

Exons 1α and 1β of I*nk4a* and A*rf* are regulated by distinct promoters, allowing for their independent transcription and epigenetic silencing. During normal mouse development, A*rf* expression is undetectable in most tissues. One notable exception is the vitreous of the eye, where transient production of $p19^{Arf}$ is important for regression of the hyaloid vasculature [7]. Hence, A*rf*-null mice are blind, but they otherwise develop normally. Yet, tumors arise spontaneously later in the life of these animals, and they are highly susceptible to cancer induction by activated oncogenes, irradiation and chemical carcinogens [8,9]. A*rf* expression is not triggered by physiologic growth-promoting signals, but its transcription ensues when proliferative signals are abnormally elevated and sustained. An engineered reporter mouse strain in which A*rf* coding sequences were substituted by a green fluorescent protein cassette placed under the control of the endogenous A*rf* promoter exhibited negligible green fluorescent protein expression in most normal mouse tissues *in vivo*. However, spontaneously appearing tumors or those activated by oncogenes, such as over-expressed Myc or mutant Ras, were brightly fluorescent [10]. Data such as these support the concept that A*rf* acts as a fuse to monitor hyper-proliferative signals. When activated by oncogenes, then, Arf diverts incipient cancer cells to either undergo senescence or apoptosis, thereby protecting the organism from tumor development.

NPM = nucleophosmin

N-terminal ubiquitination of Arf protein and its stabilization by nucleophosmin

While many investigations of A*rf* expression have focused on its transcriptional regulation, factors governing the post-translational regulation of the $p19^{Arf}$ protein were elucidated more recently. Because human $p14^{ARF}$ lacks lysine residues and mouse $p19^{Arf}$ contains only a single lysine that is not conserved among Arf proteins from different species, most researchers inferred that the ubiquitin-proteasome pathway does not mediate Arf turnover. Furthermore, due to the negligible methionine content of both the human and mouse Arf proteins, conventional "pulse-chase" experiments performed with [^{35}S]-methionine proved problematic for kinetic measurements of Arf protein turnover. However, metabolic labeling of $p19^{Arf}$ with [^{3}H]-leucine revealed that its half-life in mouse embryo fibroblasts is 6–8 hours, whether or not the cells contain the genes encoding p53 and Mdm2 [11].

Most cellular proteins are polyubiquitinated and degraded in the proteasome. Utilizing the tsBN75 cell line that carries a temperature-sensitive ubiquitin E1-activating enzyme, both $p19^{Arf}$ and $p14^{ARF}$ were found to accumulate at the non-permissive temperature [11]. Surprisingly, wild-type $p19^{Arf}$, an engineered lysine-less $p19^{Arf}$ K26R mutant, and lysine-less human $p14^{ARF}$ were each found to undergo polyubiquitination and to be degraded by the proteasome. Together, these findings implicated the primary N-terminal amino group as the site of modification. Adapting a strategy that was previously used for studying ubiquitination of an N-terminally tagged lysine-less mutant of $p21^{Cip1}$ [12], we introduced a hemagglutinin tag fused to a Factor Xa cleavage site downstream of the $p19^{Arf}$ N-terminal dipeptide. The resulting protein (Met-Gly-HA-Xa-Arf) was ubiquitinated in cells, and its digestion with Xa protease liberated the poly-ubiquitinated HA-tag, thereby providing direct biochemical evidence that $p19^{Arf}$ is ubiquitinated at its N-terminus [11].

In order for proteins to undergo N-terminal ubiquitination, the N-terminus must be unblocked. It has been estimated that about 85% of mammalian proteins are irreversibly acetylated at their N-termini during translation, a process that is largely determined by the sequence of the N-terminal amino acids [13]. N-terminal methionine is a good substrate for acetylation, but when followed by any of the seven amino acids with smallest radii of gyration, it is removed by methionine aminopeptidase [14]. Following methionine cleavage, serine and alanine are the most frequently acetylated N-terminal residues, whereas glycine is the poorest acceptor. The efficiency of N-terminal acetylation is also greatly affected by the penultimate amino acid residue; acidic residues promote acetylation, whereas basic residues are inhibitory. This suggested that methionine cleavage from the $p19^{Arf}$ Met-Gly-Arg N-terminus would yield an unblocked Gly-Arg N-terminus. This was confirmed by performing a coupled *in vitro* transcription/translation reaction in the presence of [^{3}H]-acetyl CoA. When $p19^{Arf}$ was re-engineered to contain consensus sequences that would ensure efficient N-terminal acetylation, its *in vivo* ubiquitination was partially impaired, and this correlated with increased protein stabilization [11].

Because of its highly basic composition, $p19^{Arf}$ must be "buffered" in order to function at the neutral intracellular pH of living cells. Recently, several laboratories including our own found that nucleophosmin (NPM/B23) binds to Arf with high stoichiometry within high molecular weight nucleolar complexes [15-17]. Enforcing NPM over-expression stabilized $p19^{Arf}$; conversely, reducing NPM levels with short hairpin RNAs accelerated the rate of $p19^{Arf}$ degradation [11]. In addition, hypomorphic Arf polypeptides carrying mutations in six conserved residues within the N-terminal region of the protein or lacking other short N-terminal segments were profoundly impaired in NPM binding and were intrinsically unstable with half-lives of only 1.5–2 hours. This suggests that NPM functions as a buffering chaperone for Arf proteins in cells. Although unstable Arf mutants that do not interact with NPM were also polyubiquitinated at their N-termini, it is unclear whether this is a prerequisite for their proteasomal degradation. Indeed, it is conceivable that these mutant Arf proteins are misfolded and intrinsically unstable, whereas wild-type Arf proteins, when bound to NPM, may require ubiquitination for their release and subsequent turnover.

Arf induces sumoylation

Surprisingly, Arf protein expression enhances global protein sumoylation within the nucleus of cells and targets a number of Arf-interacting proteins, including Mdm2 and NPM, for sumoylation [18-20]. In turn, Arf mutants defective in their ability to bind to Mdm2 and NPM were unable to stimulate their sumoylation. In contrast to the ubiquitin pathway, the enzymes that catalyze addition of the small ubiquitin-like modifier SUMO to proteins have been far less well studied. The sumoylation pathway includes one heterodimeric E1 and only one recognized E2 enzyme (Ubc9), and the identity and number of E3 ligases are poorly defined [21,22]. The avian adenovirus CELO Gam1 protein specifically inhibits the SUMO E1-activating enzyme and leads to the subsequent degradation of Ubc9, thereby blocking sumoylation [23]. Enforced expression of Gam1 blocked Arf-induced sumoylation of NPM and Mdm2, whereas a functionally inactive Gam1 mutant was without effect [20]. These findings suggest that Gam1 acts "upstream" of $p19^{Arf}$ in the SUMO cascade, and they raise the possibility that $p19^{Arf}$ might function as a component of a SUMO E3 ligase, possibly as a specificity factor that targets bound substrates for sumoylation.

As yet, the roles, if any, of these Arf-induced modifications in mediating the tumor-suppressive functions of Arf are unknown. Results to date suggest that the ability of Arf to enhance the sumoylation of Mdm2 may not be required for it to inhibit Mdm2's E3 ligase activity, since Arf can induce a p53 response even when sumoylation of Mdm2 is inhibited by Gam1 [20]. Still, we cannot preclude more subtle effects of Arf-induced sumoylation on the p53 transcriptional program. Moreover, we speculate that p53-independent roles of Arf might well depend upon this process, and that the identification of other Arf-induced sumoylated targets might provide new clues about these activities.

HA = hemagglutinin

The recent characterization of a karyotypically normal subtype of human acute myeloid leukemia that expresses mutant forms of NPM might potentially yield insights into Arf's modulation of cellular sumoylation. Mutations in the C-terminal region of NPM were described in approximately one-third of adult AML patients [24] and in a smaller fraction of pediatric cases [25]. These mutations create a C-terminal nuclear export signal that facilitates the efficient relocalization of the mutant NPM protein from the nucleolus into the cytoplasm [26]. Because the cytoplasmic NPM variants (designated NPMc) form hetero-oligomers with the wild-type NPM protein, NPMc acts dominantly to recruit both the wild-type NPM protein and $p19^{Arf}$ to the cytoplasm [27]. There are two main biological consequences. First, under conditions in which the expression of NPMc and endogenous NPM were equivalent (as they should be in AML cells bearing a single mutant allele), Arf activation of p53 was partially attenuated, and Arf's ability to arrest the cell cycle was blunted. Secondly, expression of NPMc ablated Arf-induced sumoylation of wild-type NPM, the mutated NPMc protein, and Mdm2, despite the ability of $p19^{Arf}$ to physically interact with each of these potential substrates. Therefore, we suspect that other components of the Arf-induced sumoylation cascade are not mobilized together with Arf and NPMc to the cytoplasm.

The road ahead?

Observations that a unique genomic organization intimately links two distinct tumor suppressor genes, *Arf* and *Ink4a*; that *Arf* responds to oncogene activation in triggering a p53 response; that $p19^{Arf}$ has both p53-dependent and -independent functions; that the regulated destruction of $p19^{Arf}$ is mediated by N-terminal ubiquitination; that $p19^{Arf}$ induces sumoylation; and that $p19^{Arf}$ can in turn be regulated through its interactions with NPM – have all provided unexpected insights into fundamental biological processes. Elucidating the regulatory mechanisms that govern Arf expression, its various activities, and its role as a tumor suppressor continues to be a journey that promises future surprises.

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AML = acute myeloid leukemia
NPMc = cytoplasmic NPM variants

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