

Addenda

A smARF Way to Die

A Novel Short Isoform of p19ARF is Linked to Autophagic Cell Death

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Addendum to:

A Short Mitochondrial Form of p19(ARF) Induces Autophagy and Caspase-Independent Cell Death

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ABSTRACT

We recently revealed a novel mechanism by which p19ARF can induce cell death. We found that the p19ARF mRNA encodes an additional shorter isoform from the same open reading frame, named smARF. smARF is a short lived protein, which is rapidly degraded by the proteasome, but accumulates after inappropriate proliferative signals generated by oncogenes. Surprisingly, smARF translocates to the mitochondria, impairs the structure of the mitochondria, and dissipates the mitochondrial membrane potential in a p53 and Bcl-2 family independent manner, ultimately inducing type II caspase-independent autophagic cell death.

The INK4A/ARF locus, a frequent target of inactivation in human cancers, encodes two distinct tumor-suppressor proteins which function as cell cycle regulators: p16INK4A¹ and the alternative reading frame protein, named p19ARF in rodents² and p14ARF in human. Alternate promoters regulate the independent production of these two mRNAs. While p16INK4A activates the Retinoblastoma protein (Rb) by inhibiting the cyclin D-dependent kinases, p19ARF activates p53 through inhibition of its regulator Mdm2. p19ARF inhibits Mdm2 by sequestering it to the nucleolus^{3,4} and by inhibiting its E3 protein ubiquitin ligase activity.^{5,6} Likewise, p19ARF was recently shown to inhibit a second p53 E3 ubiquitin ligase protein, named ARF-BP1/Mule.⁷ The p19ARF-mediated stabilization and activation of p53 ultimately turns on either the caspase-dependent apoptotic pathways or cell cycle arrest. p19ARF possesses p53-independent anti-proliferative functions as well:⁸ it can induce p53-independent apoptosis,⁹⁻¹² and it can activate p53-independent cell cycle arrest by inhibiting ribosomal RNA maturation, via its physical interactions with NPM/B23.¹³ Additionally, p19ARF can inhibit proliferative signals by inhibiting transcription factor activity.¹⁴⁻¹⁶

Recently, we discovered that this unique INK4A/ARF locus encodes an additional shorter isoform of p19ARF named smARF (short mitochondrial ARF).¹⁷ An internal AUG (Met45) in the p19ARF mRNA initiates the translation of this shorter isoform. Notably, although smARF and full length p19ARF share the same coding region frame, they differ in intracellular localization, protein stability, regulation and function. The full-length p19ARF is composed of 169 amino acids, is highly basic and hydrophobic, localizes mostly to the nucleoli,⁴ and is a relatively stable protein (half life 6-8 h).¹⁸ The first 37 amino acids of p19ARF are critical for its nucleolar localization, p53 activation and its effect on inhibition of ribosomal biogenesis. Since the smARF coding region starts at Met 45, it lacks all these known functional domains of p19ARF. Surprisingly, unlike the nucleolar p19ARF, smARF translocates to the mitochondria (Fig. 1). Biochemical cell fractionation showed that it localizes to a proteinase K-resistant compartment of the mitochondria. Under normal conditions it is rapidly degraded by the proteasome (half life less than 1 h), thus it is not harmful to the cells. Forced expression of this short isoform leads to structural damage of the mitochondria and induces dissipation of the mitochondrial membrane potential in a p53- and Bcl-2 family-independent manner. The mitochondrial outer membrane is not impaired since no marks of cytochrome c release into the cytosol could be detected and caspases were not activated. Ultimately, smARF induces massive autophagy and caspase-independent cell death.

Autophagy has been traditionally considered only as a survival mechanism. However, it has been recently recognized, that under certain conditions, especially when the apoptotic pathway is blocked, cells can use the basic autophagic machinery to kill themselves.¹⁹ Thus, the same players, upon different regulatory circuits, can accomplish different tasks. Notably, knocking down two key players in the autophagic machinery, Atg5 or Beclin-1 attenuated the ability of smARF to induce cell death, implying that the massive autophagy

observed here does not serve to protect the cells from death, but rather it serves to degrade the cells from inside leading to their death. Thus, smARF induces type II autophagic cell death.

The rapid turnover of smARF and consequent low protein levels most likely restrain its cytotoxic properties in the cell. However, inappropriate proliferative signals generated by proto-oncogenes, such as c-Myc and E2F1 can elevate both p19ARF^{20,21} and smARF.¹⁷ Thus, in principle, two pathways can be activated. p19ARF can activate a pathway from within the nucleolus which involves inhibition of ribosomal biogenesis and p53 activation, leading to cell cycle arrest or type I apoptotic cell death. smARF, on the other hand, can induce a second pathway that initiates within the mitochondria, which ultimately will induce type II autophagic cell death (Fig. 2). It remains to be determined whether these two pathways act simultaneously, or whether the nucleolar pathway should be downregulated in order for the smARF pathway to predominate.

Notably, although the different ARF orthologues share only a limited degree of homology in their amino acid sequence, most of the aforementioned functions of the nucleolar ARF were reported to be conserved in evolution. We found a similar functional conservation with respect to smARF. The human p14ARF orthologue (only 45% identical to the mouse orthologue over the region of overlap)²² is also capable of producing the novel short form of ARF. The latter (named hsmARF) is similarly produced by a mechanism of internal initiation of translation from a methionine located in the same position as that in the mouse protein. The hsmARF localizes to the mitochondria, is rapidly degraded by the proteasome and dissipates the mitochondrial membrane potential when stabilized in cells. Interestingly, rat ARF does not contain an internal Met, but still can produce a shorter form that is rapidly degraded by the proteasome,¹⁷ most probably involving a mechanism other than internal translation. Thus, the mitochondrial ARF functions are preserved through evolution.

These findings reveal a novel mode of action of p19ARF to promote caspase-independent cell death. Additional experiments are required to further gain insight into this novel pathway. For example, smARF does not contain a mitochondrial localization signal, therefore what directs its translocation to the mitochondria? smARF likewise, lacks a lysine residue that can be a site for ubiquitination. In that case, by what mechanism is it targeted for degradation by the proteasome? How does smARF cause dissipation of the mitochondrial membrane potential? How does smARF cause autophagic cell death? Are the damage to the mitochondrial structure and/or the dissipation of the mitochondrial membrane potential necessary events to initiate autophagy in this pathway? Recently, it was shown that overexpression of p19ARF can induce sumoylation,²³ but the physiological significance of this is still unclear. It would be interesting to check whether smARF is also able to sumoylate mitochondrial proteins, and if so, to examine whether it is important for its mitochondrial and autophagic functions. So far, only oncogenes were shown to elevate both p19ARF and smARF expression levels. Are there signals or specific cellular settings that differentially activate mitochondrial smARF but not the nucleolar p19ARF? Finally work has to be done to determine whether smARF-induced autophagy plays a part in suppression of

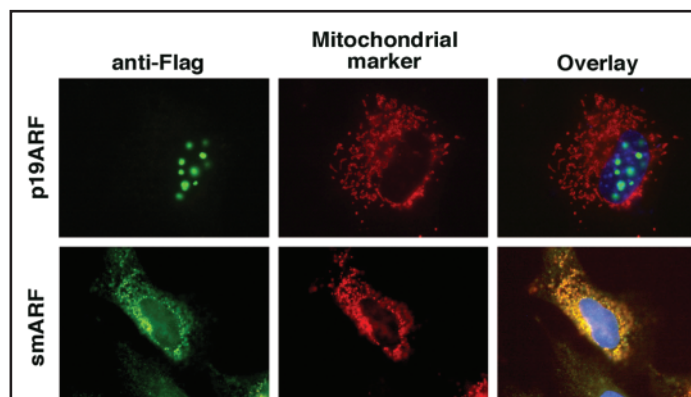


Figure 1. The differential intracellular localization of the two isoforms of ARF. Cells transiently transfected with Flag-tagged p19ARF exhibit the conventional nucleolar localization, while those transfected with Flag-tagged smARF show the unpredicted mitochondrial localization.

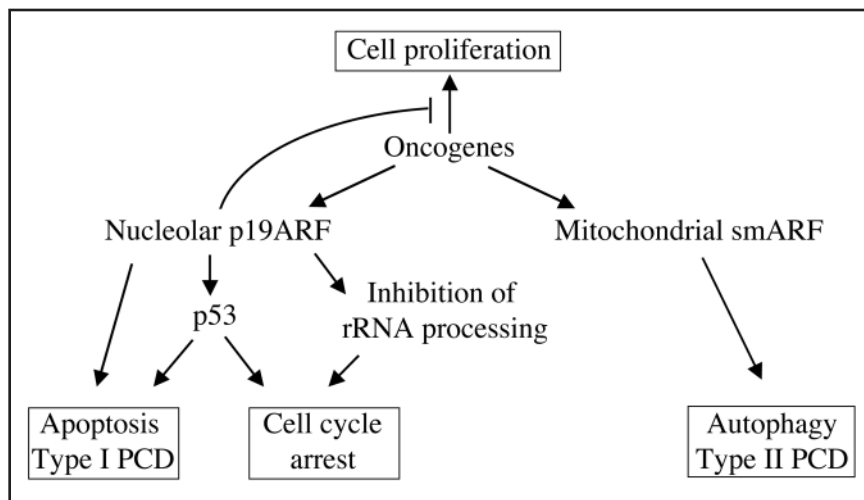


Figure 2. Possible mechanisms by which oncogenes function within cells. Oncogenes can accelerate cell proliferation; however, as a fail-safe mechanism they will also accelerate two distinct pathways that counteract cellular proliferation, growth and survival. The first pathway involves the nucleolar p19ARF protein, acting dependently or independently of p53, to induce type I caspase-dependent apoptotic cell death or cell cycle arrest. The second novel pathway relies on the mitochondrial smARF protein, which initiates type II autophagic cell death.

tumorigenesis especially in animal model systems. This requires the development of knock-in mice, which lack the nucleolar p19ARF and exclusively express smARF. Answers to this wide repertoire of molecular and functional questions will significantly advance our understanding of the complexity of the pro-death function of the ARF gene.

In conclusion, smARF is a short protein with surprising functions.

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