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Review

ARF tumor suppression in the nucleolus ☆

Leonard B. Maggi Jr., Crystal L. Winkeler [1], Alexander P. Miceli [1], Anthony J. Apicelli [1], Suzanne N. Brady [1], Michael J. Kuchenreuther [1], Jason D. Weber *

BRIGHT Institute, Department of Internal Medicine, Division of Molecular Oncology, Siteman Cancer Center, Washington University School of Medicine, Saint Louis, MO, USA

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ABSTRACT

Since its discovery close to twenty years ago, the ARF tumor suppressor has played a pivotal role in the field of cancer biology. Elucidating ARF's basal physiological function in the cell has been the focal interest of numerous laboratories throughout the world for many years. Our current understanding of ARF is constantly evolving to include novel frameworks for conceptualizing the regulation of this critical tumor suppressor. As a result of this complexity, there is great need to broaden our understanding of the intricacies governing the biology of the ARF tumor suppressor. The ARF tumor suppressor is a key sensor of signals that instruct a cell to grow and proliferate and is appropriately localized in nucleoli to limit these processes. This article is part of a Special Issue entitled: Role of the Nucleolus in Human Disease.

1. The *Ink4a/Arf* locus

The human *Ink4a/Arf* (*Cdkn2a*) locus encodes for both the cyclin-dependent kinase inhibitor $p16^{INK4A}$ and the $p14^{ARF}$ tumor suppressor ($p19^{ARF}$ in the mouse) (Fig. 1). Located on human chromosome 9 (syntenic to mouse chromosome 4), the locus also contains *Ink4b* (also known as *Cdkn2b*), which lies upstream of *Arf* and *Ink4a*. *Ink4b* is its own genetic entity, while *Ink4a* and *Arf* share two of their three exons [1,2]. It is also worth noting that a non-coding RNA, ANRIL (also known as *Cdkn2b* antisense or *Cdkn2bas*), has recently been discovered at the *Ink4b–Arf–Ink4a* locus. It has been proposed that ANRIL regulates the expression of the locus [3]. Due to splicing events, unique promoters, and unique first exons, the transcription products of *Ink4a* and *Arf* contain distinctive first exons (*Ink4a* is encoded by exon 1α and *Arf* is encoded by exon 1β) but identical second and third exons. The shared exons result in almost 70% sequence homology at the DNA level. However, *Arf* is translated in an alternative reading frame, for which it is named [1]. This results in ARF and INK4A proteins that are distinct following translation. Although alternative reading frame coding is commonly seen in viral genomes for economy of space, the *Ink4a/Arf* locus represents the only known instance in a mammalian genome. Intriguingly, the chicken ARF tumor suppressor gene does not translate the spliced exon 2 sequence and thus the functional protein is derived entirely from the unique exon 1β coding sequence, forming a truncated protein, p7 [4]. Given that the exon 1β sequences are necessary and sufficient for all of ARF's known functions [5–7], others have suggested that the evolution of the locus has allowed for this peculiar arrangement in order to provide splicing and polyadenylation sites or alternatively, to allow for coordinated transcriptional control over two tumor suppressors operating at the nexus of the critical p53 and Rb pathways [8,9].

1.1. Regulating the Arf locus

Under normal conditions, it is important to keep *Arf* (and other members of the locus) repressed (Fig. 1). Polycomb group (PcG) proteins accomplish this task. PcG proteins repress the expression of specific gene sets through extensive chromatin modifications [10]. PcG silencing occurs through the activity of diverse multiprotein complexes, Polycomb repressive complex 1 or 2 (PRC1 or PRC2, respectively) [11]. The complexes are extremely diverse in composition, but in general, PRC2 contains the histone methyltransferase EZH2, which together with other components is responsible for the trimethylation of histone H3 on Lys 27 [12]; specific members of PRC1 can then recognize the $H3K27me^3$ mark with the chromodomain of a particular PcG component [10]. One of the main PcG components that repress *Arf* expression is B lymphoma Mo-MLV insertion region 1 (BMI-1) [13]. As its name implies, BMI-1 is a proto-oncogene that cooperates with Myc to promote the generation of B- and T-cell lymphomas [14,15]. *Bmi-1*-null MEFs undergo premature senescence due to the marked upregulation of ARF and $p16^{Ink4a}$; overexpression of BMI-1 drastically decreases the expression of ARF and $p16^{Ink4a}$ as well [16]. Of note, BMI-1-repression of the *Ink4a/Arf* locus is mechanistically responsible for BMI-1's

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* Corresponding author at: BRIGHT Institute, Department of Internal Medicine, Division of Molecular Oncology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8069, St. Louis, MO 63110, USA. Tel.: +1 314 747 3896; fax: +1 314 362 0152.
E-mail address: jweber@dom.wustl.edu (J.D. Weber).
[1] These authors contributed equally.

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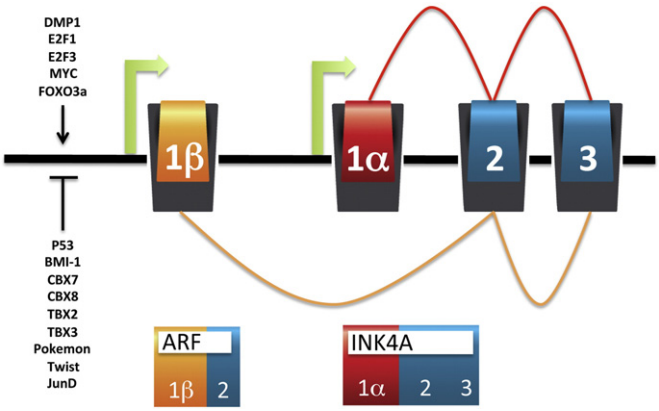

Fig. 1. The *Ink4a/Arf* locus. The locus contains two unique exon 1s and shared exons 2 and 3. The *Arf* promoter is repressed by numerous transcription factors and complexes. Oncoproteins activate *Arf* transcription. Translation of *Arf* mRNAs occurs in an alternate reading frame, resulting in an ARF protein that is completely different from INK4a.

collaboration with Myc in tumorigenesis [17]. Additionally, CBX7 is another chromodomain containing PcG protein that reduces the expression of *Ink4a/Arf*, through a manner independent of BMI-1 [18]. CBX8, another chromodomain-PcG protein that acts in PRC1, decreases the expression of the *Ink4a/Arf* locus [19]. Moreover, PcG-mediated gene silencing is the molecular mechanism through which p53 can repress *Arf* expression. Zeng et al. suggest that p53 can bind *Arf*'s promoter and recruit histone deacetylase complexes (HDAC) and PcG proteins [20]. The loss of HDAC and PcG-mediated repression is the reason why ARF protein levels increase in the absence of *Trp53* [20]. However, it should be noted that in the face of oncogenic stimuli ARF levels rapidly increase, arguing for the necessity of the PcG regulatory factors that repress *Arf* expression. Indeed, the histone demethylase JMJD3 can oppose the activity of EZH2-containing PRC2 complexes, resulting in derepression of *Ink4a/Arf* expression in wild type MEFs [21]. Similarly, the chromatin remodeling SWI/SNF complex family member, SNF5, contributes to the activation of ARF in response to Ras^{V12} in murine muscle tissues [22].

Yet, PcG-complexes are not the sole repressors of *Arf* gene expression. Disruption of E2F-repressive complexes in MEFs increases the expression levels of ARF [23]. Moreover, E2F3b is largely responsible for downregulating *Arf* expression because loss of E2F3b is sufficient to de-repress ARF expression and induce p53 and p21 [24]. This study also indicates that the transcriptional activating complexes, E2F1 and E2F3a, are recruited to the *Arf* promoter and displace E2F3b to promote *Arf* expression [24]. Other transcriptional repressors that lower *Arf* expression include Pokemon, Tbx2 and Tbx3 [25–27], although the precise molecular mechanism governing their regulation of *Arf* remains to be fully elucidated.

1.2. Arf loss in cancer

$p16^{INK4a}$ and ARF have synergistic tumor suppressive functions as mice containing loss of both are more tumor prone than those with the loss of only one or the other [28]. Mice disrupted for only exon 1β develop tumors as early as eight weeks. After one year, 80% of the mice die from spontaneous tumor development, with a mean survival latency of 38 weeks. Heterozygous mice also develop tumors, albeit after a longer latency compared to $Arf^{-/-}$ mice. Upon examination of $Arf^{+/-}$ mice, tumor formation is accompanied by loss of the remaining allele. The tumor spectrum in $Arf^{-/-}$ mice includes sarcomas (43%), lymphoid malignancies (29%), carcinomas (17%), and tumors of the nervous system (11%) [29]. Additionally, $Arf^{-/-}$ mice are also susceptible to accelerated tumor formation caused by 7,12-dimethylbenz-α-anthracene (DMBA) [29,30]. Mouse embryonic fibroblasts taken from $Arf^{-/-}$ mice are immortal and transformed upon the ectopic expression of oncogenic Ras^{V12} [30]. This last observation is of great importance because it suggests that loss of *Arf* can substitute for Myc in classical Myc- and Ras-transformation assays [31]. Loss of *Arf* synergizes with other genetic alterations to exacerbate the severity of tumorigenesis. *Arf* loss enhances the aggressiveness observed in Bcr-Abl induced acute lymphoblastic leukemia [32]. Also, loss of *Arf* in thymocyte derived *Notched1*-induced T-cell acute lymphoblastic leukemia generates a marked increase in disease onset and penetrance [33]. Similar findings have also been reported in Ras^{V12}-driven skin papillomas and carcinomas [34]. Most strikingly, $Arf^{-/-}$ mice expressing the *Eμ-Myc* transgene, succumb to their B-cell lymphomas within eleven weeks of life [35]. Taken together, these data clearly demonstrate the significance for ARF's physiological role as a robust tumor suppressor.

In human cancers, one of the most frequent cytogenetic events is the homozygous loss of the *Ink4b–Arf–Ink4a* locus [31,36–38]. In fact, the frequency of mutation at this locus is second only to the *p53* locus [39,40]. In most cases of human cancer, all three proteins of the *INK4b–Arf–INK4a* locus are lost, making it difficult to determine their individual roles in human tumor suppression. In these situations, it is impossible to appreciate the relative contribution of ARF's specific tumor suppression against the incipient tumorigenesis. Additionally, we cannot surmise whether the selective pressure to inactivate the locus is in response to a single member of the locus or to the combinatorial tumor suppressive functions of *Ink4b*, *Arf*, or *Ink4a*. Mutations within exon 2 that affect both ARF and $p16^{Ink4a}$ are found in cancers [41–45]. However, there are specific examples in which only *Arf* appears to be affected in human cancer, and these cases appear to be most common in melanoma patients. Gene deletions in families with melanoma-neural system tumor syndrome occur specifically in exon 1β [46]. Deletion of exon 1β happens in members of a family predisposed to melanoma [47]. Splice mutations arise in exon 1β that facilitate *Arf* haploinsufficiency in a family with melanoma and breast cancer [48]. In addition to melanoma cases, nine of fifty glioblastoma patients have a specific deletion of *Arf* [49]. Aside from deletions, mutations of exon 1β that impair ARF function are seen in a case of melanoma [50]. Furthermore, the *Arf* promoter contains a CpG island, and ARF expression is consequently downregulated by promoter methylation [51–57]. Saporita et al. [31] describe the vast nature of ARF-specific alterations in a wide spectrum of human cancers, including: anaplastic meningioma [58], angiosarcomas [59], Barrett's adenocarcinoma [60], bladder cancer [61], breast cancer [62–65], chronic myeloid leukemia [66], colorectal carcinoma [67,68], ependymoma [69], epithelial ovarian cancer [70], gastric cancer [71], osteosarcoma [72], salivary gland carcinoma [73], T-cell acute lymphoblastic leukemia [41], and Wilm's Tumor [74]. Taken together, this collective wealth of evidence clearly demonstrates the importance of ARF tumor suppression in human cancers.

1.3. Arf transcription and translation

Oncogenic signals are persistent and obligate attributes of cancer cells that evolve due to the selective mitogenic advantage they bestow onto the incipient tumor cell. However, an intrinsic tumor suppressive mechanism that could thwart the tumorigenic potential of these stimuli would be at the forefront of the cell's barriers against tumor formation. In fact, it is at this interface where ARF exerts its robust tumor suppressive function in the cell (Fig. 2). *Arf* transcription is upregulated in response to a host of oncogenic signals including c-Myc, Ras, E2F-1, E1A, and v-Abl [38].

In vivo support of ARF's induction in response to oncogenic signals was derived utilizing an *Arf* reporter mouse. Here, green fluorescent protein (GFP) is knocked into the endogenous *Arf* locus, and is therefore subject to the transcriptional regulation that would induce *Arf* expression [75,76]. Of note, MEFs isolated from $Arf^{+/GFP}$ and $Arf^{GFP/GFP}$ mice recapitulate the findings that *Arf* is responsive to oncogenic Ras^{V12} in vitro. Importantly, spontaneous tumors, as well as X-ray induced tumors, develop in $Arf^{GFP/GFP}$ mice within the observed kinetics of