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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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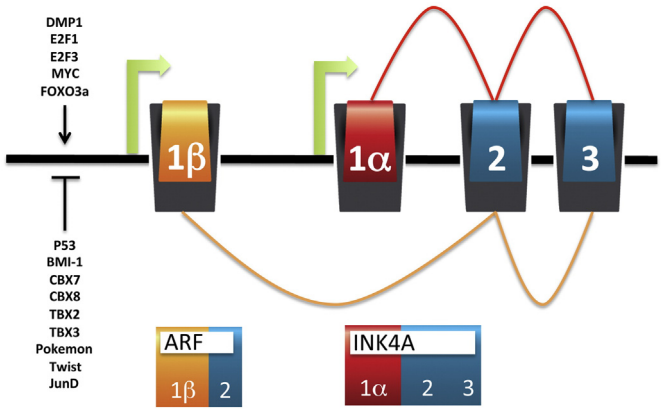

Fig. 1. The *Ink4a/Arf* locus. The locus contains two unique exon 1s and shared exons 2 and 3. The *Arf* promoter is repressed by numerous transcription factors and complexes. Oncoproteins activate *Arf* transcription. Translation of *Arf* mRNAs occurs in an alternate reading frame, resulting in an ARF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

Fig. 2. The many functions of the ARF tumor suppressor. In response to oncogenic stress, ARF can bind to proteins that positively regulate cell cycle progression. Through this mechanism, ARF restrains cell proliferation and triggers apoptosis. During development, basal ARF regulates testes maturation and regression of the hyaloid vasculature. A major p53-independent function of ARF is to monitor and regulate ribosome output. This is accomplished through numerous nucleolar ARF interactions that prevent rRNA transcription, rRNA processing, and nuclear export of ribosomes. ARF also prevents angiogenesis by limiting the translation of existing VEGFA mRNAs.

Arf $^{-/-}$ mice, which is expected since these mice lack a functional ARF protein [75,76]. GFP expression and fluorescence are routinely detected within the lymphomas and sarcomas that developed in these mice [75,76].

This led to a search of the *Arf* promoter for known binding sites of transcription factors. The ARF tumor suppressor contains a canonical DMP1 binding site, 5′-CCCGGATGC-3′, within its promoter [77]. The DMP1 transcription factor is a likely candidate for *Arf* regulation given that it is known to arrest mouse fibroblasts upon overexpression, and human *Dmp1* is frequently deleted in myeloid leukemia [78,79]. DMP1 binds and activates the *Arf* promoter. Moreover, infection of wild type MEFs with DMP1 induces ARF expression and cell cycle arrest. Importantly, in the absence of *Arf*, DMP1 overexpression has no effect on the cell cycle, indicating that DMP-1-induced arrest is dependent upon ARF [77]. DMP1 is also a key mediator of Ras-induced ARF expression [80]. However, Ras-induced ARF protein expression is only mildly attenuated in the absence of *Dmp1* [81,82]. By signaling through the Ras/PI3K/TSC/mTOR pathway, ARF induction in *Dmp1*-null cells occurs in the absence of enhanced *Arf* transcription [83]. We now know that ARF is upregulated both transcriptionally and translationally in response to oncogenic Ras to induce cell cycle arrest.

Interestingly, Ras-induced ARF-mediate cell cycle arrest is not immediate. Wild-type MEFs transduced with oncogenic Ras^{V12} accumulate ARF protein over time and do not succumb to ARF-mediated cell cycle arrest for approximately 5 days [84]. While increases in both ARF transcription and translation can be quickly detected upon Ras^{V12} overexpression in wild-type MEFs, this data suggests that a threshold level of ARF protein must accumulate before cell cycle arrest can be achieved. Given the potent nature of ARF-mediated cell cycle arrest [30,76,84], this makes sense as it allows the cell to achieve growth and proliferate before immediately blocking it with cell cycle arrest. While proliferation is necessary, ARF can accumulate over a prolonged growth cycle to prevent unchecked cellular growth.

1.4. ARF's structure, cellular location, and stabilization

The structure of ARF is important to consider when studying the protein's localization, stabilization, and binding partners. Mouse ARF ($p19^{ARF}$) contains 169 amino acids, while human ARF ($p14^{ARF}$) contains 132. Of this relatively small protein, nearly 20% of the residues are arginines, making ARF a highly basic protein. The basic nature of ARF renders it highly insoluble and is likely the reason for its lack of structure [85]. Moreover, this property also renders ARF a very "sticky" protein, which makes it difficult to discern which of its proposed binding partners is physiologically relevant. It is likely that ARF requires constant binding with another protein to bring its charge to a more neutral pH in order to function in vivo [1,86,87]. In fact, owing to a nucleolar localization signal, ARF is typically found within nucleoli bound in high molecular weight complexes with other proteins [88]. In consideration of ubiquitination on lysine residues, mouse ARF contains only one lysine (Lys26) while human ARF has none. ARF has a half-life of about 6 h and is ultimately destroyed by ubiquitin-mediated proteasomal degradation. However, the ubiquitin moiety is not added to the sole lysine in mouse ARF as removal of that lysine still results in ARF's degradation. Instead, both mouse and human ARF undergo N-terminal ubiquitination, which signals them for destruction [89].

2. p53-dependent ARF tumor suppression

p53 has been labeled the "*guardian of the genome*". *TP53* is mutated in approximately half of all human cancers [90]. The genetic alterations in *TP53* are frequently missense mutations that disrupt p53's ability to act as a transcriptional activator [91]. The p53 tumor suppressor is a key sensory molecule that regulates a plethora of downstream targets capable of triggering cell cycle arrest, apoptosis, senescence, DNA repair, and autophagy in response to robust oncogenic stimulation, DNA damage, and other cellular stressors [92]. Given the potent effects of p53 induction on cell proliferation and viability, it is essential to keep *TP53* expression under tight modulation.

One crucial level of regulation involves the RING-finger containing E3 ligase termed Mouse Double Minute 2 (MDM2 or HDM2 in humans) whose direct interaction with p53 blocks p53-mediated transactivation [93] and targets the p53 protein for proteosomal degradation [93–95]. MDM2 also disrupts p53 function as a transcription factor by binding to p53's transactivation domain and interfering with the recruitment of basal transcription machinery [96]. This protein interaction plays an important role in keeping the basal cellular levels and activity of p53 low enough to avoid interference with cell cycle progression and cell survival. Furthermore, a negative feedback loop exists whereby p53 binds specifically to the *Mdm2* promoter and stimulates its transcription [97]. This is critical to terminate the p53-mediated signaling response. The importance of the MDM2–p53 interaction is underscored by work demonstrating that *Mdm2* $^{-/-}$ mice are embryonic lethal but are rescued by concomitant deletion of *p53* [98]. Negative regulators of p53 function, such as MDM2, are classified as proto-oncogenes and lead to constitutive inhibition of p53 thereby promoting cancer without a need to alter the *p53* gene itself [99]. Thus, it is important that additional tumor suppressors are present to ensure that the negative regulators of p53 are inhibited.

ARF's classical role as a tumor suppressor involves p53 activation (Fig. 2). When prompted by oncogenic signals, ARF's N-terminal domain (amino acids 1–14) associates with the central region of MDM2, a region separate from MDM2's p53 binding domain, its nuclear import or export domains, and its E3 ligase domain [6,100,101]. This interaction sequesters MDM2 in the granular region of the nucleolus, a membraneless dynamic subnuclear organelle where ARF typically resides [102]. This subcellular re-localization event is dependent on both ARF's nucleolar localization signal (NoLS) as well as a cryptic NoLS within MDM2 that is exposed when these two proteins are bound to one another [103]. The sequestration of MDM2 by ARF prevents the binding of

MDM2 to p53 and the ability of MDM2 to shuttle between the nucleus and cytoplasm, thereby impeding its ability to transport p53 to the cytoplasm for degradation [104]. Keeping in mind that *Arf* transcription is negatively regulated by p53 as highlighted earlier, yet another negative feedback loop exists to limit p53 activation.

The elegant "supra p53" mouse model study addresses the importance of ARF in p53's tumor suppressive role in response to oncogenic cues [105]. Mice carrying an extra copy of p53 are completely protected from oncogenic stress-induced tumorigenesis. However, this protection is completely abrogated in *Arf*-deficient "supra p53" mice. This study also highlights the fact that ARF tumor suppression is activated in response to oncogenic stress and not DNA damage.

3. p53-independent functions of ARF

Do ARF and p53 act in a linear pathway? Somewhat surprisingly, *Arf/p53* double-knockout (DKO) and *Arf/p53/Mdm2* triple-knockout (TKO) mice present multiple tumors of distinct origins, namely the simultaneous formation of carcinomas and lymphomas within the same animal that are not observed in either *Arf*-null or *p53*-null animals [7]. If ARF only exerts its tumor suppressive functions through p53, we would expect DKO and TKO mice to display the same types of tumors as *p53*-null mice. Furthermore, an *Arf* mutant lacking amino acids 1–14, which are necessary for ARF's ability to bind MDM2, cannot arrest TKO cells [7]. Thus, the amino terminal 14 amino acids of ARF are necessary and sufficient for both its p53-dependent and -independent tumor suppressive functions.

3.1. Sumoylation and ribosomal RNA processing

The nucleolus has long been appreciated as the site of ribosomal RNA (rRNA) transcription and assembly into mature ribosomal subunits [106–109]. Given the nucleolar localization of ARF, it may function as an inhibitor of ribosomal biogenesis. Overexpression of ARF dramatically interferes with ribosomal RNA processing (Fig. 2 and [31]). This effect is independent of p53 as *p53/Arf* double-null cells transduced with p53 fail to alter ribosome biogenesis [110]. ARF might function in the nucleolus by binding to and inhibiting an rRNA biogenesis factor.

Recent data [88,111,112] indicates that this may be the case. Using a variety of affinity-purification approaches followed by mass spectrometry to identify co-precipitating proteins, several labs including our own identified nucleophosmin (NPM), an abundant 37-kDa nucleolar phosphoprotein as a binding partner for ARF. NPM has been shown to be required for proper rRNA processing in vitro [113,114]. However, while in vivo evidence of a direct role for NPM in rRNA processing is lacking, its role in ribosome biogenesis has been demonstrated [115] and reviewed in [116]. NPM is reported to be a potent oncogene [117] and a transcriptional target of MYC [118,119], as well as having a myriad of other nucleic-acid binding activities [120]. As a nucleocytoplasmic shuttling protein, NPM is also thought to function as a chaperone for other protein complexes that are exported from the nucleus [120–123].

Using an ARF-inducible cell line, upregulation of ARF led to the sumoylation of MDM2 and NPM [124]. Whereas the ARF–MDM2 complex clearly exists only in a p53-dependent setting, ARF can interact with NPM in both p53-dependent and p53-independent contexts [112,124]. Concomitant with a rise in ARF expression, nucleolar SUMO (small ubiquitin-like modifier)-reactive species accumulates. ARF mutants lacking the MDM2 and NPM binding region or the nucleolar localization signal fail to induce the sumoylation of MDM2 and NPM [124].

In a second study, utilizing the same ARF-inducible cell culture system, ARF induction hinders ribosomal RNA (rRNA) processing, specifically impairing the processing of the 47/45S and 32S precursors, which is evidenced by the accumulation of improperly processed rRNA intermediates [110]. Importantly, overexpression of p53 fails to inhibit rRNA processing, pointing to a specific role for ARF in this process [110]. Finally, ARF's ability to impair rRNA processing is strictly dependent upon its evolutionarily conserved N-terminal 14 amino acids (residues 1–14) [110], the region required for ARF's p53-dependent and p53-independent pathways of growth arrest [7].

DDX5 is another target through which ARF participates in the regulation of ribosome biogenesis in a p53-independent manner [125]. DDX5 is a member of the DEAD-box family of RNA helicases that is involved with many cellular functions through its ability to unwind RNA duplexes and remodel RNP complexes [126]. DDX5 enhances the synthesis and processing rRNA through a mechanism modulated by ARF. ARF inhibits the ability of DDX5 to localize within the nucleolus, where DDX5 executes its pro-growth activity [125]. An intriguing component of this analysis is the finding that DDX5 activity is required for the anchorage independent growth in soft agar for Ras^{V12}-transformed $Arf^{-/-}$ MEFs, which highlights the necessity for ribosome biogenesis in cellular transformation [125]. A similar mechanism is noted by Lessard et al., who demonstrate that ARF can control ribosome biogenesis by regulating the subnuclear localization of RNA polymerase I transcription factor, TTF-1 [127]; ARF inhibits the nucleolar import of TTF-1 from the nucleoplasm, consequently repressesing rRNA transcription. While ARF's role in dampening rRNA processing is executed independently of its engagement with MDM2 and the p53 tumor suppressor pathway, evolution may have utilized ARF to coordinately regulate proliferation and ribosome biogenesis within the confines of the nucleolus [110].

3.2. Other ARF binding partners

To date, over 30 ARF-interacting proteins have been reported in the literature, including viral proteins (e.g., HPV16E7, TBP1), nuclear/nucleolar proteins (NPM, nucleolin, NIAM), DNA modifying enzymes (e.g., WRN, Topoisomerase I), posttranslational modifying enzymes (e.g., Mdm2, ARF-BP1, ATR, ATM, UBC9), transcriptional repressors (e.g., BCL6, p120E4F) and transcriptional activators (e.g., p53, Myc, E2F1, DP1, HIF1α) [87]. It may be that not every protein within this rapidly expanding collection is a true physiological and functional target of ARF, especially considering ARF's extraordinarily basic charge and potential for promiscuous binding of proteins when grossly overexpressed in cells. As detailed below, understanding the basal functions of ARF in the cell in the absence of oncogenic stimuli may provide a context to help elucidate the purpose of these seemingly disparate ARF binding partners so far described in the literature.

MYC is an oncogenic transcription factor widely overexpressed in a variety of cancers and its activity is necessary for proper cell cycle progression. As such, it serves as an important target for a number of tumor suppressor pathways; indeed it is already implicated in the ARF–MDM2–p53 axis as hyperactive MYC is a potent inducer of ARF expression [128]. Somewhat surprisingly, both human and mouse ARF directly interact with MYC on chromatin and antagonize its transactivation activity but not its ability to repress certain loci [129–131]. This interaction does not interfere with MYC's binding to its heterodimerization partner, MAX, nor does it affect cell cycle arrest in cells lacking *p53*. Published accounts of this interaction differ on mechanism (ARF-dependent re-localization of MYC to the nucleolus vs. MYC-dependent re-localization of ARF to the nucleoplasm), which may be reflective of the relative amounts of overexpressed protein [129,132].

Like MYC, E2F1 is implicated in promoting ARF transcription [133,134], as well as having its transactivation activity inhibited by ARF [135–138]. Furthermore, some E2F isoforms also antagonize *Arf* transcription under basal states, ensuring that levels of ARF remain low enough to prevent inappropriate p53-mediated cell cycle arrest. Such regulation is abolished (i.e. ARF expression is de-repressed) upon overexpression of activating E2F (such as E2F1) or inactivation of RB [23,24,139].

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ARF inhibits the function of E2F complexes by binding to the E2F protein [135,136] or its dimerization partner, DP1 [137,138], and preventing the formation of active complexes. Although in most reports, the binding of ARF to either E2F1 or DP1 is accompanied by re-localization of the proteins from the nucleoplasm to the nucleolus [136,137], the inhibition of E2F1's transcriptional activity need not depend on nucleolar sequestration [135]. These processes are p53-independent in that co-transfection of E2F1 and ARF into *p53*$^{-/-}$ MEFs inhibits E2F1's transactivation activity [135]. Furthermore, induction of ARF in a U2OS cell line engineered to stably express a dominant negative mutant p53 still causes reduction of mRNA levels of the E2F1 target, cyclin A, prior to S-phase arrest. Knockdown of ARF via targeted lentiviral shRNA interference in *Mdm2*$^{-/-}$p53$^{-/-}$ MEFs leads to accumulation of cyclin A mRNA as well as enhanced promoter occupancy of *DHFR* (another E2F1 target) by DP1 as demonstrated by chromatin immunoprecipitation [138]. Taken together, these data indicate that ARF antagonizes E2F1 function in a p53-independent manner.

UBF is the rate-limiting component of the basal transcription factor for PolI transcription of rDNA, and its regulation is tightly controlled by phosphorylation by members of the PI3K–Akt–mTOR pathway. ARF binds to UBF in vitro and in vivo, and suppresses its transcriptional activity, leading to a decrease in 47S precursor in cells induced to overexpress ARF. Furthermore, endogenous ARF co-localizes with UBF in the granular component of the nucleolus away from the site of rDNA transcription (the dense fibrillar compartments), and also co-immunoprecipitates with UBF. Induction of ARF results in a decrease in the amount of phosphorylated UBF, indicating that ARF may either physically block the interaction of UBF with its upstream kinases or prevent accession to UBF by kinases that may be restricted to the rDNA loci, as ARF improperly sequesters UBF in the granular region of the nucleolus [140]. ARF also associates with topoisomerase I. ARF stimulates Topo I's relaxation activity of supercoiled DNA both in vitro and in vivo. ARF and Topo I co-immunoprecipitate in both HeLa and 293 extracts, and overexpression of ARF and Topo I in Saos2 cells (which have low levels of ARF) results in their co-localization in the granular component of the nucleolus [141].

Like MYC and E2F, FOXM1b is also a transcription factor necessary for cell cycle progression, especially in hepatocytes. It is a member of the forkhead box (Fox) family that shares homology in the winged helix DNA-binding domain [142]. Transgenic mice overexpressing Foxm1b exhibit accelerated cell cycle entry in regenerating hepatocytes [143–145], and mice with a specific deletion of Foxm1b in the liver are resistant to the onset of hepatocellular carcinoma (HCC) following carcinogen exposure [146]. In wild type mice, ARF is robustly expressed in hepatocytes six weeks after treatment with the carcinogen DEN/PB, but this expression is lost in adenomas that began developing 23 weeks after treatment. Considering that *Foxm1b*$^{-/-}$ hepatocytes are resistant to the effects of DEN/PB in vivo, ARF might antagonize FOXM1b function, thereby protecting against carcinogen induced HCC. Consistent with this idea, ARF co-immunoprecipitates with FOXM1b, thereby impairing FOXM1b's transactivation activity. Moreover, in normal hepatocytes after exposure to DEN/PB, but not in liver adenomas lacking ARF expression, Foxm1b immunostaining is observed in the nucleolus, suggesting that ARF might also act to sequester FOXM1b to inactivate it, similar to the MDM2, MYC and E2F [146].

Additionally, ARF's implicated role in the regulation of gene expression extends beyond transcriptional mechanisms. ARF suppresses the translation of vascular endothelial growth factor A (VEGFA) mRNA in the absence of p53 [147]. VEGFA is a key mediator of angiogenesis because VEGFA stimulates the growth of new blood vessels from adjacent microvessels [148]. Importantly, loss of *Arf* alters the distribution of *Vegfa* transcripts along actively translating polyribosomes without affecting the transcription of *Vegfa* mRNA [147]. Similar findings are seen for Drosha, a RNase III endonuclease involved in the processing of rRNA and microRNAs [149]. In the absence of *Arf*, the association of *Drosha* mRNA with polysomes is enhanced, causing increased levels of Drosha protein expression [149]. Similar to the data observed for the DDX5, Drosha activity is required for the transformation *Arf*$^{-/-}$ MEFs by Ras^{V12} [149].

4. Role of basal nucleolar ARF

The basal expression level of ARF is relatively low in a normal proliferating cell. It is for this reason that some presume that these low amounts of ARF have no particular cellular function, and that only in the face of oncogenic stimuli do elevated levels of *Arf* gene expression assume a physiological role. While ARF is primarily recognized as a protein upregulated in the face of oncogenic stress, there is data suggesting important cellular roles for basal ARF. It is becoming increasingly clearer that even though ARF is nearly undetectable in many cells, it plays an integral role based on studies analyzing the effects of its loss.

4.1. Basal ARF regulates nucleolar structure and function

Given the nucleolar localization of ARF and its interaction with NPM, basal ARF might maintain nucleolar structure and limit protein synthesis [150]. *Arf* loss results in an increase in both the number and size of silver-stained nucleolar organizing regions (AgNORs) in mouse embryonic fibroblasts [150]. AgNORs highlight argyrophilic proteins that surround nucleoli. An increased AgNOR index is associated with poor prognoses in cancer [151] and, thus, this data suggests that ARF maintains the structure and likely function of proteins within nucleoli. In situ AgNOR staining on tissues from *Arf*$^{-/-}$ mice corroborates this data. Both intestine and liver tissues exhibit an increase in total AgNOR area in the absence of *Arf* [150]. In low-passage MEFs, *Arf* loss also enhances protein synthesis as assessed by ^{35}S-methionine incorporation, resulting in an increase in both protein content and cell volume [150]. Importantly, enhanced protein synthesis in these cells is independent of proliferation, as the total cell number does not increase over seven days. Again, the increases in protein synthesis upon *Arf* loss are supported by in vivo results demonstrating that *Arf* loss in liver tissue also causes an increase in protein synthesis by ^{35}S-methionine incorporation [150]. Loss of *Arf* also results in a significant increase in newly transcribed 47S transcripts. In accordance with previously published data, ARF overexpression impedes processing of the 47S rRNA into the 32S rRNA intermediate [110,150]. The final step of ribosome biogenesis is the export of the ribosomal subunits. By radioactively labeling the rRNA subunits with ^{3}H-methyl methionine, *Arf* $^{-/-}$ MEFs export ribosomal subunits into the cytoplasm at a faster rate than that observed in wild-type cells [150]. This result is in accordance with previously published data showing that ARF interacts with NPM, which is known to be important for shuttling ribosomes from the nucleus to the cytoplasm [115,152,153]. Importantly, *Arf* loss amplifies each of the three steps in ribosomal biogenesis: transcription, processing, and export.

4.2. The role of ARF in mouse eye development

Initially, *Arf*$^{-/-}$mice develop normally despite the fact that their eyes are slightly smaller compared to the eyes of wild type mice [29,30,154]. Upon closer examination, McKeller and colleagues noticed that *Arf* $^{-/-}$ mice had a funnel-shaped mass of cells in the vitreous of their eyes just behind the lens. Wild type mice are born with elements of the hyaloid vascular system (HVS), including endothelial cells, perivascular cells forming the hyaloid artery, and several other types of perivascular cells. Normally, the HVS will regress by postnatal day 14 [155]. Although the HVS was still present in *Arf* $^{-/-}$ P10 mice, the authors did not detect any cellular components of the HVS by postnatal day 10 in wild type mice [154]. Regression of the HVS is important for normal eye development; failed regression results in a human eye disease known as persistent hyperplastic primary vitreous or PHPV and results in microphthalmia (abnormally small eyes) [156,157]. Beginning at P14, *Arf* $^{-/-}$ mice display both defects in the neurorentina

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and the lens, which ultimately results in blindness [154]. Importantly, these characteristics are not observed in *p53*$^{-/-}$ mice, indicating that the role of ARF in hyaloid vascular regression is independent of p53 [154].

4.3. The role of ARF in male germ cell development

In addition to ARF expression within the eye, ARF is highly expressed in one other normal cell: male spermatogonia [76,158]. In mice, spermatogenesis occurs within the first month of life. Spermatogonia are cells that line the basement membrane of each seminiferous tubule; these are the cells that express ARF [158]. *Arf* $^{-/-}$mice display reduced sperm number compared to wild type mice due to an increase in apoptosis during germ cell development [159,160]. Notably, there is no increase in the proliferation of spermatogonia during germ cell development upon *Arf* loss [160]. While the apoptosis of these cells is dependent upon p53, the functions of ARF that regulate apoptosis are independent of p53. Cells void of *Arf* display increased levels of phosphorylated histone H2AX [160]. H2AX is normally phosphorylated at the leptene stage of meiosis, but disappears by early pachytene upon synapsis of homologous chromosomes [161,162]. Importantly, deletion of *p53* is unable to rescue the defect in H2AX phosphorylation [160]. Taken together, the role of ARF in male germ cell development is counterintuitive; ARF actually *prevents* p53 from inducing apoptosis in primary spermatocytes [160].

5. Conclusions

A variety of oncogenic proteins have co-opted control of translation and ribosome biogenesis as a means to further the growth of malignant, rapidly dividing cells by providing them with an unregulated supply of ribosomes primed to churn out the necessary proteins to promote proliferation. However, the cell is not without defense against such aberrant activities; the ARF tumor suppressor directly interferes with proliferation through p53 activation and ribosome biogenesis through its nucleolar interactions. Furthermore, given its nucleolar topology and sensitivity to hyperproliferative signals, the ARF tumor suppressor protein is uniquely positioned to inhibit such activity, both through its ability to induce p53-dependent cell cycle arrest, and its other p53-independent functions in the nucleolus.

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