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ABSTRACT

ARF is an alternative reading frame product of the *INK4a/ARF* locus, which is inactivated in numerous human cancers. ARF is a key regulator of cellular senescence, an irreversible cell growth arrest that suppresses tumor cell growth. It functions by sequestering MDM2, a p53 E3 ligase, in the nucleolus, thus activating p53. Besides MDM2, ARF has numerous other interacting partners that induce either cellular senescence or apoptosis in a p53-independent manner. This further complicates the dynamics of the ARF network. Expression of ARF is frequently disrupted in human cancers, and its cause has been mainly attributed to epigenetic and transcriptional regulation. Vigorous studies on various transcription factors that either positively or negatively regulate ARF transcription have been carried out. However, recent focus on posttranslational modifications, particularly ubiquitination, indicates wider dynamic controls of ARF than previously known. In this review, we discuss the role and dynamic regulation of ARF in senescence and cancer.

INTRODUCTION

The tumor suppressors p15^{ink4b}, p14^{ARF}, and p16^{ink4a}, are well-characterized products of the *INK4b/ARF/INK4a* gene locus. Corresponding to the region of mouse chromosome 4, this locus is situated on chromosome 9 of the human genome within a 35 kb region. As expected for potent tumor suppressors, the *INK4b/ARF/INK4a* locus is frequently targeted for deletion or epigenetic suppression in numerous cancers. The coding regions of p14^{ARF} and p16^{ink4a} start from exons 1 β and 1 α , respectively. Moreover, owing to an alternative reading frame, they comprise of completely different amino acids despite sharing exons 2 and 3. Although p15^{ink4b} has a similar role in cell cycle inhibition, it has a coding region physically separated from that of p14^{ARF} and p16^{ink4a} (Figure 1) (1, 2).

ARF's major role as a tumor suppressor is intimately related to p53 stabilization, which induces cellular senescence and prevents tumor cell growth. ARF releases p53 from MDM2, a well-known E3-ubiquitin ligase of p53, by trapping MDM2 in the nucleolus via direct interaction, which physically separates p53 from MDM2 (3-6). ARF's interactions with protein partners other than MDM2 also result in suppression of tumor growth via induction of either cellular senescence or apoptosis in a p53-independent manner. These have been evidenced using p53-deficient cell lines and knockout (KO) mice models. Moreover, recent identification of posttranslational regulatory mechanisms further complicates the dynamics of the ARF mechanism (7-9). In this review, we discuss the transcriptional as well as posttranslational regulatory mechanisms of ARF, in an attempt to provide comprehensive understanding of the same.

REGULATION OF ARF

Transcriptional regulation of ARF

Since the emergence of ARF as a tumor suppressor, the regulation of its expression has been one of the most pursued areas of research. Numerous transcription factors (TFs), which regulate ARF either positively or negatively, have been discovered (Figure 2). In particular, the E2F1 transcription factor (regulates genes involved in cell cycle regulation) induces ARF transcription by directly interacting with its binding site upstream of the exon 1 β , thus activating apoptosis and cell growth arrest (10). This is one of the most well characterized pathways of oncogene-induced senescence, activated upon oncogenic stress. However, this process is differently modulated by different E2F isoforms. For instance, E2F3b, an isoform of E2F3, represses ARF transcription and stimulates cellular growth. The observation that the loss of ARF can rescue E2F3b depletion-mediated cell growth arrest clearly suggests anti-reciprocal correlation between these two proteins (11). Myc, another well-established and vigorously investigated oncogene, exhibits regulatory patterns similar to E2F1. To prevent hyper-proliferation of cells under oncogenic stress, it also activates fail-safe programs such as apoptosis and cellular senescence by inducing ARF transcription (12). Frederique and colleagues reported that Myc overexpression induces accumulation of ARF both at the mRNA and protein levels in mouse embryonic fibroblasts (MEFs) (13). They showed that Myc-induced ARF activates p53 signaling thus preventing immortalization of the MEFs. Further, Myc also activates FoxO-mediated ARF transcription. Caroline and colleagues reported that Myc signaling increases nuclear FoxO, which in turn binds to the ARF promoter and thus suppresses Myc-driven lymphomagenesis in mice (14). A cyclin D-binding Myb-like protein, DMP1, also induces ARF. Kazushi and colleagues demonstrated that DMP1 binds to

the ARF promoter and activates its transcription in MEFs, which results in cell cycle arrest (15). Furthermore, the ARF promoter also provides several binding sites for acute myeloid leukemia-1 (AML1), which activates its transcription leading to cellular senescence in MEFs. However, the t(8;21) fusion protein AML1-ETO, which is frequently expressed in acute leukemia, represses ARF expression (16). Meanwhile, oncogenic signaling by RAS influences ARF expression in a DMP1-dependent manner. RAS signaling induces Jun-mediated DMP1 transcription thus increasing ARF expression (17). Next, Yanbin and colleagues suggested that TGF β signaling directly induces ARF expression. They found that TGF β signaling elevates the ARF mRNA levels through Smad2/3 and p38 MAPKs in MEFs. Smad2/3 is known to bind directly to the ARF promoter upon stimulation by TGF β . However, it is yet to be addressed how Smad and p38 MAPK signaling cooperate to induce ARF expression (18). p38 MAPK has previously been reported to regulate ARF expression. Dmitry and colleagues found that decreased Wip1 phosphatase increases ARF expression in a p38 MAPK-dependent manner in Ppm1d^{-/-} MEFs (19). Moreover, a recent study suggests that HKR3 (Human Krüppel-related 3) activates ARF transcription by binding to the ARF promoter with coactivator p300, which induces acetylation of the histones H3 and H4 (20).

Meanwhile, several repressors of ARF transcription have also been reported (Figure 2). For example, the polycomb group gene BMI-1 suppresses cellular senescence through repression of ARF transcription. BMI-1-deficient MEFs show impaired cell cycle progression and enter premature senescence, which can be rescued by ARF depletion (21). BMI-1 also requires the EZH2-containing Polycomb-Repressive Complex 2 (PRC2) to repress ARF transcription. PRC2 maintains the levels of H3K27Me3 as well as the BMI-1/PRC1 complex at the ARF locus (22). Another polycomb group gene, CBX7, is known to increase the

lifespan of normal human cells and MEFs through suppression of ARF expression, independent of BMI-1 (23). Further, TBX2 immortalizes MEFs and decreases senescence in normal human cells by repression of ARF transcription (24). The basic helix-loop-helix (bHLH) transcription factor Twist-1 is also known to activate the recruitment of EZH2 to the ARF transcription start site. Thus, it increases the levels of H3K27Me3 on the ARF locus, followed by repression of ARF transcription (25).

Although many factors that regulate the transcription of ARF are well described, its posttranslational regulation is largely unknown. Recently, some posttranslational regulators of ARF have been reported, thus magnifying its importance in senescence and tumorigenesis.

Posttranslational regulation of ARF

Although the functional importance of ARF in cellular senescence and tumor suppression is well characterized, knowledge about its posttranslational regulation is limited. A previous report suggested that ARF, which has no lysine sites, is polyubiquitinated at its N-terminus followed by proteasomal degradation by unknown an E3-ubiquitin ligase (26). Five years later, the first E3-ubiquitin ligase for ARF, TRIP12, was identified and named ULF (ubiquitin ligase for ARF) (Figure 3) (7). ULF induces polyubiquitination and proteasomal degradation of ARF, thus activating cell proliferation. Interestingly, the ULF-mediated degradation of ARF is further regulated by NPM and c-Myc, suggesting that Myc regulates ARF both transcriptionally and translationally. Subsequently, Ko and colleagues reported a second E3-ubiquitin ligase, Makorin 1 (MKRN1), which targets ARF. MKRN1 KO MEFs presented retarded cell growth and senescence with concomitant increase in the ARF protein levels (8). Corroborating these results, MKRN1 was also shown to induce ubiquitination and proteasomal degradation of ARF. Moreover, its ablation decreased tumor growth through

induction of the ARF-dependent senescence in xenograft models using p53-positive and -negative gastric cancer cell lines. Further, tumor tissues from gastric cancer patients showed negative correlation between MKRN1 and ARF proteins. Siva1 has also been reported as an E3-ubiquitin ligase for ARF. It induces proteasomal degradation of ARF, thus inhibiting p53 function (9). The existence of several E3 ligases for ARF is intriguing. Systemic studies on the cellular localization of these E3 ligases might help to shed some light. It is also possible that each ligase is a mediator that links the different signaling pathways involving ARF. Extensive studies on each ligase are required for the complete elucidation of ARF regulation.

ARF IN SENESCENCE AND CANCER

Tumor suppressive function of ARF

Senescence is an irreversible cell growth arrest that prevents hyper proliferation of cancer cells. As oncogenic signal stimulation always carries the risk of cancer cell eruption, normal cells require senescence as a fail-safe program to prevent hyper proliferation and tumorigenesis. ARF plays a key role in this fail-safe program through activation of the p53 pathway (Figure 4). ARF binds and sequesters MDM2 in the nucleolus leading to the activation of p53 in the nucleoplasm (3-6). The activated p53 then promotes transcription of numerous target genes such as BAX or p21, thus inducing either cellular senescence or apoptosis (27-29). Mice lacking p19ARF are susceptible to early tumors. In fact, ARF and p53 double KO mice show tumor incidences similar to those with p53 KO alone. This suggests that the tumor suppressive function of ARF is entirely p53-dependent (30).

While there is no argument on the dependency of ARF on p53, some evidence shows

that ARF might have a p53-independent tumor suppressive function as well. Weber and colleagues reported that triple KO (TKO) mice for MDM2, p53, and p19ARF show greater frequency of multiple tumors than do double KO mice for MDM2 and p53. Moreover, reintroduction of ARF into TKO MEFs induces cell cycle arrest. These results suggest that ARF has an additional tumor suppressive role that is p53/MDM2-independent (31). Genetic deficiency in ARF but not p53 accelerates development of melanoma in a genetically engineered mouse model, while senescence is induced in p53- but not in ARF-deficient primary melanocytes (32). The induction of cell cycle arrest, cellular senescence, apoptosis, and regression in xenograft tumors by ARF in p53-deficient cell lines indicates obvious p53-independent roles for ARF (33-38). Possibly, p53-independent functions of ARF are carried out through its interactions with numerous regulatory proteins (Figure 4). For example, Tip60 directly binds to and induces acetylation of RB, leading to its destabilization through proteasomal degradation. ARF prevents the Tip60-mediated RB acetylation and leads to accumulation of hyper phosphorylated RB, which then triggers anti-proliferative functions (39). Further, through MALDI-TOF analyses, NPM was also identified as a binding partner of ARF. ARF binds and sequesters NPM in the nucleolus thus blocking its nucleocytoplasmic shuttling, and resulting in subsequent cell growth arrest (40, 41). ARF also inhibits the activities of various TFs such as E2F1, HIF-1 α , Foxm1, c-Myc and n-Myc through direct interaction, thus preventing cell proliferation (33, 42-45). This shows the negative feedback regulation of ARF induced by some of these TFs. ARF also induces the ATR/CHK1-dependent RelA (NF-kB) phosphorylation and reduces its transcriptional activity (46). Furthermore, CtBP1 and 2, transcription co-repressors that have anti-apoptotic functions, are also reported to interact with ARF. Binding of ARF to CtBP1 and 2 promotes their

proteasomal degradation (47). In addition, ARF also stimulates the sumoylation of several target proteins including NPM, MDM2, WRN, and Miz-1 (48-52). While its mechanism remains unclear, the observation that ARF interacts with UBC9, a sumo E2 conjugating enzyme, indicates that ARF might facilitate sumo transfer from the E2 complex to its target proteins (52). In addition, there are many other proteins that interact with ARF (such as HPV16E7, HSP70 and others) and assist in its p53-dependent or -independent functions (53, 54).

In short, ARF has numerous interacting partners other than MDM2, which facilitate and maximize its tumor suppressive effect via induction of cellular senescence or apoptosis in p53-dependent or -independent manner (Figure 4). Given its p53-dependent and -independent functions, elaborate studies on the context-dependent roles of ARF are required, since most cancer cells are either deprived of p53 or carry mutant p53.

ARF KO mice

The first KO mice for ARF were developed even before it was found that *INK4a* and *ARF* coexist on the same genomic locus. As a result, the first KO mice had both the *INK4a* and *ARF* sites eliminated. These mice spontaneously developed various types of cancers within the first year. KO MEFs also displayed significantly faster growth rate than wild type MEFs. Furthermore, they were transformed by oncogenic stimulus of RASV12 (55). Later, mice lacking p19ARF but not p16INK4a were also generated by targeting exon 1 β . These mice also developed numerous tumors- spontaneously as well as upon carcinogen treatment, leading to death within the first year. In several mouse strains (C57BL6, 129svj X C57BL6 and FVB), ARF-specific KO mice develop sarcoma, squamous cell carcinoma, lung carcinoma, and metastatic lymphoma. Moreover, exposure to carcinogens such as DMBA, X-

rays, and irradiation make ARF KO mice more prone to tumors (Table 1) (56-58). The fact that mice and MEFs lacking ARF alone show similar features of cancer development and cell growth raised curiosity about the characteristics of mice KO for Ink4a alone. In contrast to p19ARF-deficient MEFs, MEFs that lack p16Ink4a but retain p19ARF show normal cell growth features and are susceptible to RAS-induced senescence (59). Meanwhile, p16-null MEFs exhibit increased immortalization compared with wild type MEFs, though less compared with p19ARF KO and p19ARF/p16ink4a KO MEFs. KO mice lacking p16 alone also develop spontaneous and carcinogen-induced tumors, which indicate that p16Ink4a functions as a tumor suppressor in association with p19ARF.

Immunoglobulin promoter enhancer ($E\mu$)-driven Myc expression promotes B-cell lymphoma in mice. The B-cell lymphoma latency is significantly shortened in mice lacking both Ink4a and ARF, or ARF alone (27, 60, 61). Moreover, the role of the *INK4a/ARF* locus in HRASV12-induced melanoma has been reported using melanocyte-specific HRASV12 transgenic mice. Mice lacking p19ARF and p16ink4a were shown to develop melanoma with short latency and high penetrance (62).

The tumor suppressive role of Ink4a/ARF was also studied in a super Ink4a/ARF mouse strain (carrying a transgenic copy of the entire *INK4a/ARF* locus), wherein increased activities of these tumor suppressors were observed (63). Furthermore, cells derived from these mice showed increased resistance to oncogene-induced transformation. Surprisingly, the super Ink4a/ARF mice showed higher resistance to carcinogen-induced tumor development without affecting normal viability and aging. This suggests that restricted increase in the amount of tumor suppressors at systemic levels might enhance an individual's ability to resist cancer development.

Status of ARF in human cancers

Given that, ARF plays an important role in tumor suppression, its deregulation in numerous cancers is also reported (Table 2). Studies on the deregulation of ARF have focused on gene loss or silencing by promoter hyper methylation and mutation. Its expression patterns have been mainly studied at the transcript level because of its low expression, and availability constraints of its antibodies for immunohistochemistry. However, ARF protein expression in human cancers has been constantly pursued since the importance of its posttranslational regulation was postulated. Low mRNA levels of *ARF* have been frequently observed in human cancers including those of the breast, colon, and liver. This has chiefly been attributed to gene silencing by promoter methylation and deletion of the gene locus. Both homologous deletion and loss of heterozygosity for this locus have been detected in human cancers (35, 44, 64-69). These alterations mostly result in deletion of the entire *INK4a/ARF* locus affecting the expressions of both ARF and Ink4a. The best-known genetic alteration that specifically affects ARF expression is promoter hyper methylation. In this context, the *ARF* promoter is a CpG island that can be silenced by DNA hyper methylation. Silencing of the gene by this mechanism has been reported in numerous human cancers including those of the colon, liver, breast, and lung (64-66, 69-82). Hyper-methylation of the *ARF* promoter occurs independently of the *INK4a* promoter methylation, thus suggesting specific alteration of the ARF expression in these cancers. Contrastingly, elevated levels of *ARF* mRNA have also been reported in tumors (68). ARF might also be upregulated in cancers by various oncogenic stimuli such as c-Myc, RAS, and E2F1. Furthermore, mutations such as short deletions or insertions in the *ARF*-specific exon 1 β , and missense mutations in exon 2 have been frequently reported in familial melanoma syndromes (83-86).

Sylvie and colleagues reported that protein expression of ARF is low in lung cancer, and that there is frequent uncoupling between transcript and protein levels (87). Thus, they suggested that ARF is also inactivated at the posttranslational level in cancers. In a recent study, the authors also reported that the ARF protein is expressed at low levels in gastric cancer, and is negatively correlated with MKRN1 expression (an E3 ubiquitin ligase that induces ARF degradation) (8). These results also support the importance of the posttranslational regulation of ARF in tumor suppression/progression.

CONCLUSION

The role of ARF in tumor suppression is relatively well established through various experiments using cancer cells, mice models, and human patients with cancer. As is well known, the key mechanism of tumor suppression by ARF is induction of cellular senescence via activation of the p53 pathway. ARF binds and sequesters MDM2 in the nucleolus, thus preventing the degradation of p53. However, the p53-independent role of ARF is still to be clearly delineated, and its targets verified. Toward this, KO mice models have proven to be useful as shown by Weber and colleagues (discussed above). On one hand, these results led to the discovery of novel ARF interacting partners that regulate tumor suppression in a p53-independent manner. On the other, the discovery of too many interacting partners has now caused considerable confusion. ARF interacts with numerous partners and regulates their sumoylation, transcriptional activities, or protein turnover resulting in p53-independent inhibition of tumor cell growth. As p53 is frequently mutated in many cancers, a clear understanding of this p53-independent function of ARF might provide crucial clues to finding

therapeutic targets in p53-mutated cancers. To this end, the downstream and upstream factors of ARF, along with their regulatory mechanisms, must be addressed extensively. In addition, the differential posttranslational regulation of ARF associated with tumor suppression and tumorigenesis must be elucidated using mouse models and human patient samples.

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FIGURE LEGENDS

Figure 1. The *INK4b/ARF/INK4a* locus. The *INK4b/ARF/INK4a* locus encodes three proteins including p15INK4b, p14ARF, and p16INK4a. The coding regions of p14ARF and p16ink4a start in different exons—exon 1, 1□, and 1□, respectively. Exon 2 of the *INK4b/ARF/INK4a* locus is shared by *p14ARF* and *p16ink4a*. They comprise completely different amino acids despite sharing the exons 2 and 3, through an alternative reading frame.

Figure 2. Transcriptional regulation of ARF. Positive and negative regulators of ARF transcription. Transcriptional activators and repressors are shown in the upper part and lower parts, respectively.

Figure 3. Posttranslational regulation of ARF. Three ubiquitin E3 ligases have been reported. ULF, MKRN1, and Siva1 bind directly to and induce ubiquitination of ARF resulting in the induction of its proteasomal degradation.

Figure 4. p53 dependent and independent tumor suppressive functions of ARF. ARF plays a key role in cellular senescence and tumorigenesis through activation of the p53 pathway. ARF binds and sequesters MDM2 in the nucleolus leading to the activation of p53 in the nucleoplasm. p53 Leads to transcription of numerous target genes and activates cellular senescence or apoptosis. ARF also functions in a p53-independent manner through interaction with numerous target proteins. ARF regulates sumoylation, transcription activities, or protein turnover of target proteins, which lead to tumor suppression and senescence.

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Fig1. The *INK4b/ARF/INK4a* locus

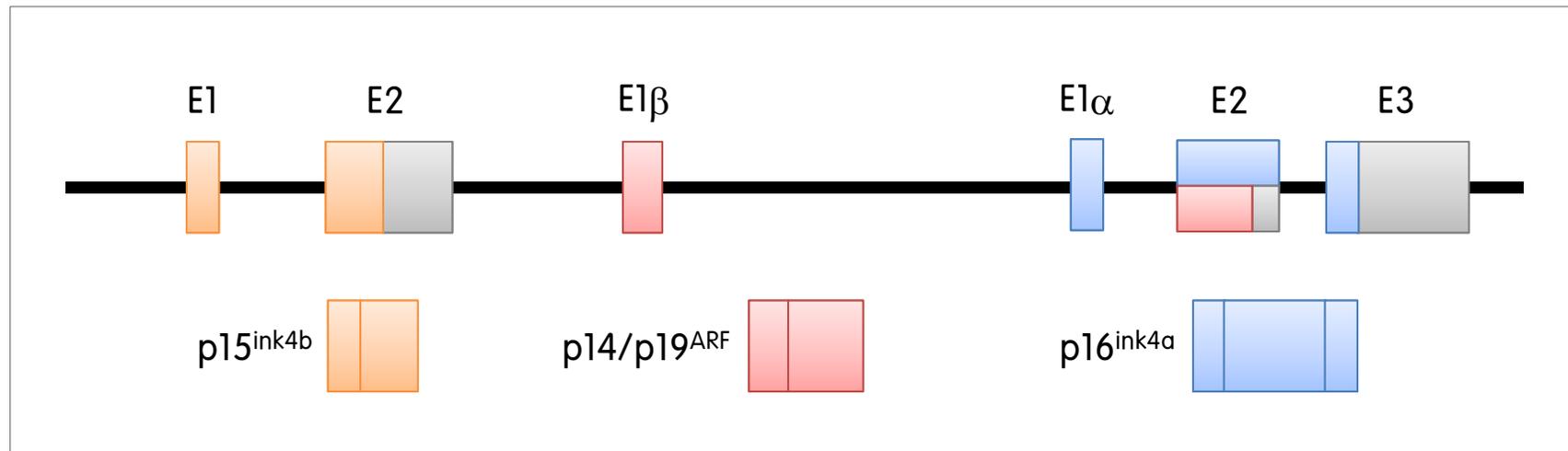


Fig2. Transcriptional regulation of ARF

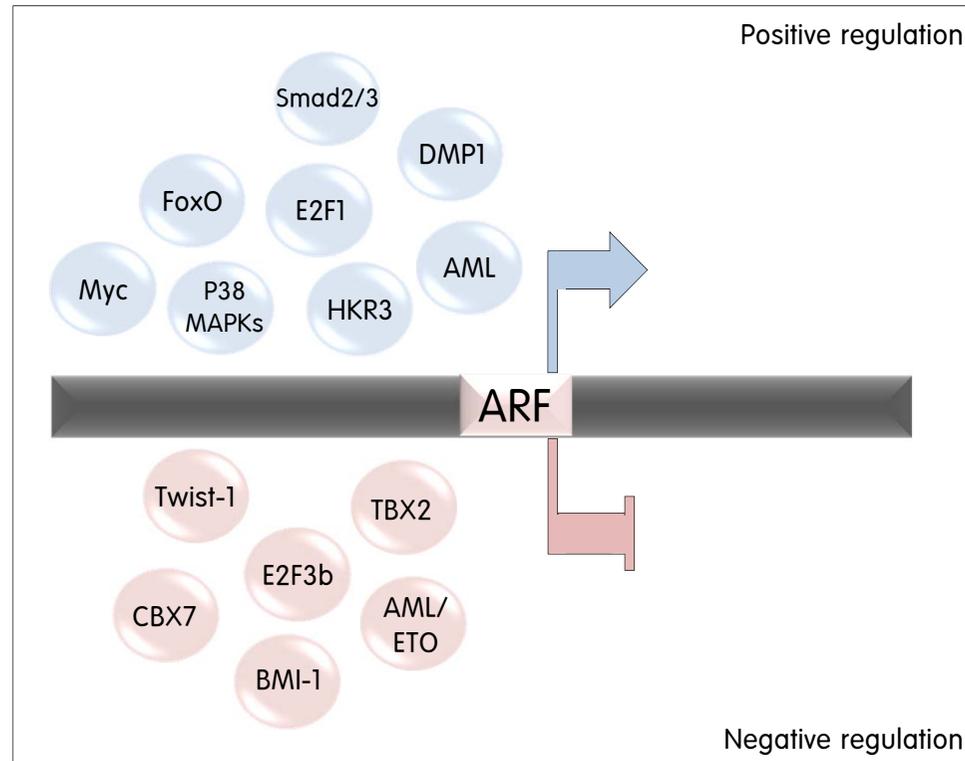


Fig3. Posttranslational regulation of ARF

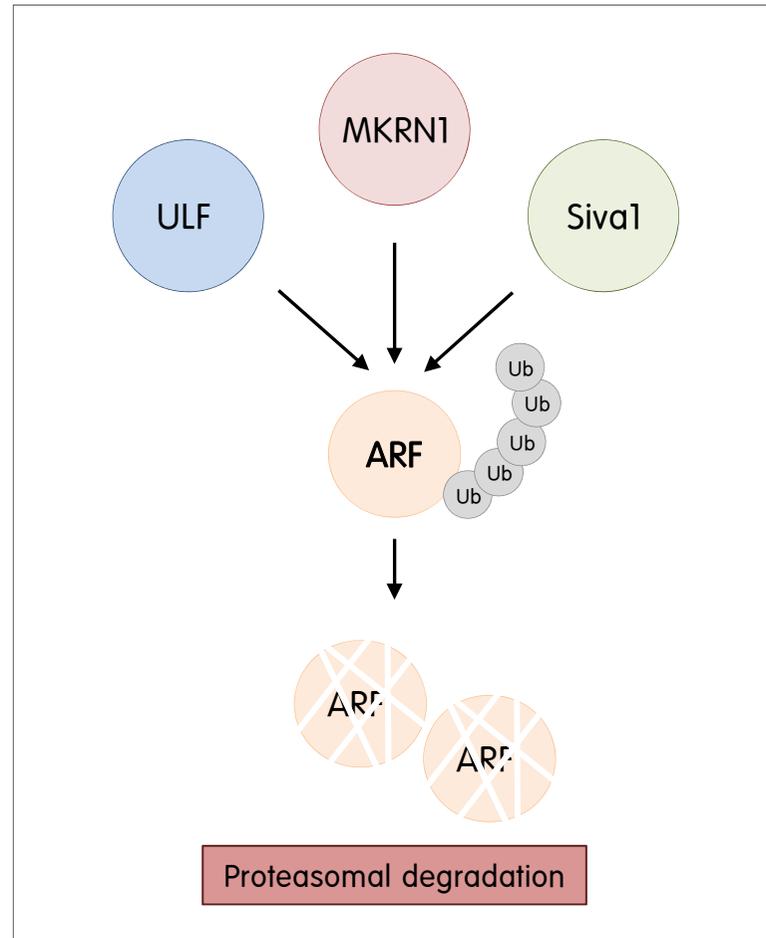


Fig4. p53 dependent and independent tumor suppressive function of ARF

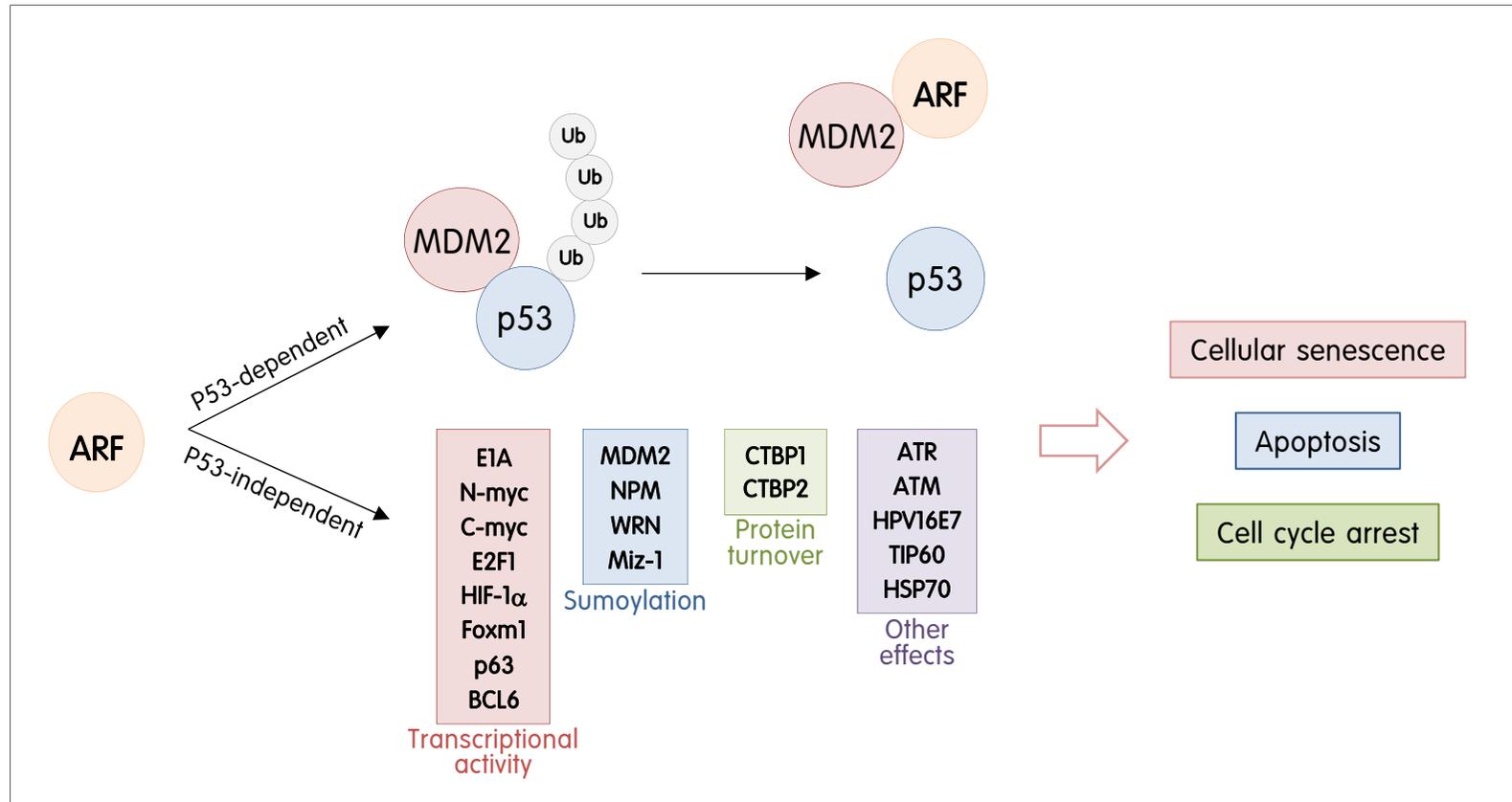


Table 1. Knockout mice of ARF and INK4a

	Strain	Targeting site	Treatment	Type of tumor formation	latency	ref
ARF ^{-/-}	129svj X C57BL6	Exon1 β	None	Sarcoma(43%) Lymphoid malignancies(29%) Carcinoma(17%) /Squamous cell carcinoma /Pulmonary adenocarcinoma /Poorly differentiated carcinoma in pancreas /Salivary gland carcinoma Tumors of the nervous system(11%) /Spinal cord neural sheath tumor /Glioma	~38 weeks	56
			DMBA	Squamous cell carcinoma /+Lymphoma /+Sarcoma and adnexal tumor /+Sarcoma and lymphomax /+sarcoma	~24weeks	
			X-ray	Sarcomas Lymphoma Meningeal tumor		
ARF ^{-/-}	FVB	Exon1 β	None	Small lymphoma(33%) Malign. Sp. Cell neo(30%) Lung carcinoma(12%) Osteogenic sarcoma(9%) Tumors of the nervous system(9%) Carcinoma/HCC(6%)	~62weeks	57
			DMBA	Small lymphoma(60%) Malign. Sp. Cell neo(40%) Lung carcinoma(15%)	~24weeks	
ARF ^{-/-}	C57Bl/6	Exon1 β	None	Fibrosarcoma(33.3%) Metastatic salivary gland carcinoma(16.7%) Thymoma(16.7%) Malignant fibrous histiocytoma(16.7%) Lymphoma(brain)(16.7%)	~21weeks	58
			DMBA	Epidermal papilloma(55.5%) Lymphoma, Epidermal papilloma Fibrosarcoma, malignant adnexal tumor Fibrosarcoma, epidermal papilloma Invasive epidermoid carcinoma	~20weeks	
			Irradiation	Fibrosarcoma(50%) Lymphoma(brain)(50%)	~19weeks	
INK4a ^{-/-}	FVB	Exon1 α	None	Soft-tissue sarcoma(12.8%) Splenic lymphoma(10.25%) Melanoma(2.56%)	~44weeks	59
			DMBA	Thymic lymphoma(13%) Splenic lymphoma(6%) Soft-tissue sarcoma(10%) Malignant spindle-cell neoplasma(6%) Lung adenoma(6%) Squamous papilloma(29%)	~23weeks	
INK4a ^{-/-} , ARF ^{-/-}	C57BL/6	Exon2, 3	None	Fibrosarcoma(33.3%) Sarcoma Liposarcoma, Lymphoma Angiosarcoma B-cell lymphoma Lymphoma	~36weeks	55
			UV	Fibrosarcoma(50%) Squamous cell carcinoma Lymphoma	~36weeks	
			DMBA+UV	Fibrosarcoma(50%) B-cell lymphoma Lymphoma Low grade Fibrosarcoma Squamous cell carcinoma	~14weeks	

Table 2. ARF status in human cancers

Tissue type	ARF status	Frequency(%)	Ref
Breast	Low mRNA	77	70
	promoter methylation	50	
	HD	11.5	
	LOH	27	
	High mRNA	17	71
	no alteration	88	
	promoter methylation	24	
Bladder	promoter methylation	56	72
	LOH	22	68
	HD	14	
	mutation	2	
Colon	promoter methylation	33	73
	promoter methylation	50.8	74
	promoter methylation	28.2	80
	promoter methylation	32	75
Liver	promoter methylation	25	76
	HD	4	
	LOH	16	
	promoter methylation	12	77
	HD	2.3	67
	mutation	4.6	
Gastric	High mRNA	93.2	78
	promoter methylation (diffuse type)	45.5	
Lung	promoter methylation (intestinal)	25	69
	promoter methylation	30	
	LOH	26	79
Oral	promoter methylation	8	66
	HD	12	
	promoter methylation	43.8	64
	HD	26.5	
Prostate	promoter methylation	6.25	65
	HD	6.25	
Kidney	HD	23.5	81
Brain	promoter methylation	22	79

HD: Homologus Deletion, LOH: Loss Of Heterozygosity